Over-expression of AMP-activated protein kinase impairs pancreatic β-cell function *in vivo*

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Abstract

Treatment of type 1 diabetes by islet transplantation is currently limited by loss of functional β-cell mass after transplantation. We investigated here whether adenovirus-mediated changes in AMP-activated protein kinase (AMPK) activity, previously shown to affect insulin secretion *in vitro*, might affect islet graft function *in vivo*. In isolated mouse and rat islets, insulin secretion stimulated by 17 (vs 3) mmol/l glucose was inhibited by 36·5% ($P<0·01$) and 43% ($P<0·02$) respectively after over-expression of constitutively-active AMPK- (AMPK CA) versus null (eGFP-expressing) viruses, and glucose oxidation was decreased by 38% ($P<0·05$) and 26·6% ($P<0·05$) respectively. Increases in apoptotic index (terminal deoxynucleotide transferase-mediated deoxyuridine trisphosphate biotin nick end-labelling) (TUNEL)) were also observed in AMPK CA- ($22·8 \pm 3·6$% TUNEL-positive cells, $P<0·001$), but not AMPK DN- ($2·72 \pm 3·9$%, positive cells, $P=0·05$) infected islets, versus null adenovirus-treated islets ($0·68 \pm 0·36$% positive cells). Correspondingly, transplantation of islets expressing AMPK CA into streptozotocin-diabetic C57 BL/6 mice improved glycaemic control less effectively than transplantation with either null ($P<0·02$) or AMPK-DN-infected ($P<0·01$) islets. We conclude that activation of AMPK inhibits β-cell function *in vivo* and may represent a target for therapeutic intervention during islet transplantation.

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Introduction

In the wake of improvements achieved by Shapiro *et al.* (2000), human islet transplantation is now considered a potentially useful treatment modality for type 1 diabetes (Korsgren *et al.* 2005). At present, however, the shortage of transplantable human islet material and the requirement for multiple donors severely limit the usefulness of this approach. This problem is further aggravated by the substantial (60–80%) and time-dependent loss of islet material that appears to occur after transplantation (Korsgren *et al.* 2005, Rickels *et al.* 2005). A similar phenomenon is observed in other species (Davalli *et al.* 1995) and probably occurs by multiple, non-immune and immune-mediated mechanisms (Korsgren *et al.* 2005). Strategies which inhibit these losses, and thus enhance β-cell survival, are therefore likely to reduce the number of islet equivalents required initially, and to extend the usefulness of islet transplantation. To this end, adenovirus-mediated introduction into islets of potentially cytoprotective genes, such as bcl-2 (Contreras *et al.* 2001), insulin-like growth factor-1 (Giannoukakis *et al.* 2000*a*), Iκ-B kinase inhibitor (Rehman *et al.* 2003), Iκ-B repressor (Giannoukakis *et al.* 2000*b*), erythropoietin (Fenjves *et al.* 2004) or a tumour necrosis factor (TNF) receptor fusion decoy (Machen *et al.* 2004), is considered an exciting approach.

Adenosine monophosphate-activated protein kinase (AMPK), an evolutionarily conserved serine/threonine kinase (Carling 2004), is ubiquitously expressed in mammalian tissues and is involved in the regulation of substrate (notably fatty acid) oxidation. AMPK is seen as a potentially interesting therapeutic target for type 2 diabetes (Ruderman & Prentki 2004), since activation of the enzyme in muscle and liver enhances glucose uptake and suppresses glucose release respectively (Zhou *et al.* 2001). Changes in AMPK activity in these tissues have thus been proposed to be involved in the anti-hyperglycaemic effects of metformin and the glitazone class of antidiabetic drugs (Fryer *et al.* 2002). On the other hand, activation of AMPK in isolated rodent and human islets, as well as clonal β cells, suppresses glucose metabolism and glucose-stimulated insulin secretion (GSIS) (da Silva Xavier *et al.* 2000, Eto *et al.* 2002, Rutter *et al.* 2003, Leclerc *et al.* 2004), and enhances β-cell death through apoptosis (Kefas *et al.* 2003*b*) (F Diraison and G A R, unpublished data). Importantly, the extent to which AMPK activation in the β cell limits the otherwise beneficial effects of the

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above antihyperglycaemic agents is presently unexplored (Rutter *et al.* 2003).

The role of AMPK in the regulation of insulin secretion is largely untested *in vivo*. Thus, while whole-body inactivation of the α2-isoform of the catalytic subunit by homologous recombination in mice (Viollet *et al.* 2003) leads to abnormal glucose tolerance, this appears largely to be the result of increased sympathetic tone. Importantly, islets isolated from α2-knockout mice display no evident abnormalities in glucose-stimulated insulin secretion *ex vivo* (Viollet *et al.* 2003), consistent with the predominant expression of α1 in islets and β cells (da Silva Xavier *et al.* 2000). Moreover, compensatory increases in AMPKα1 activity in β cells cannot be ruled out in the α2 knockout mouse model.

The present study was undertaken with two aims in mind:

1. to clarify the role of AMPK in the control of insulin secretion and β-cell mass *in vivo*

2. to assess the therapeutic potential of islet-specific regulation of the enzyme in a rodent model.

We demonstrate that adenovirus-mediated overexpression of an activated form of AMPK (Woods *et al.* 2000) markedly inhibits the improvement of glycaemic control achieved by transplantation of islets under the kidney capsule of streptozotocin-diabetic syngeneic mice, demonstrating the potential importance of AMPK in controlling functional β-cell mass *in vivo*.

Materials and Methods

Adenoviral preparation

Adenoviruses expressing the constitutively active NH_2-terminal fragment common to mammalian AMPKα1 and α2 (AMPK1–312,T^{172}D; Ad-AMPK CA), and the full-length AMPKα2 mutated in the active site (D^{157}A; Ad-AMPK DN) or enhanced green fluorescent protein alone (Ad-eGFP) were generated as described (da Silva Xavier *et al.* 2003) by subcloning the corresponding cDNAs into pAdTrackCMV (He *et al.* 1998). Viruses were amplified in HEK293 cells and purified as described on CsCl gradients (Ainscow & Rutter 2001).

Animals

Inbred male C57 BL/6 mice aged 8–14 weeks (20–25 g) were used as donors and transplant recipients. All experimental mice were bred and housed in the specific-pathogen-free facility, University of Bristol. They were housed at 22 °C with 12-h light/dark cycle and *ad libitum* access to chow and water. Procedures were performed in accordance with UK Home Office regulations, and the NIH 'Principles of Laboratory Animal Care' were followed throughout. Male Wistar rats (220–250 g) were supplied by the University of Bristol Medical School Animal Facility.

Islet isolation

Donor mice were killed by cervical dislocation and underwent laparotomy to expose the common bile duct. The duct was then clamped at its insertion into the duodenum and cannulated proximally with a 27-gauge needle and 3 ml collagenase P solution (1 mg/ml, Sigma) in Hanks' buffered salt solution (HBSS; Gibco) supplemented with 0·01 M HEPES, pH 7·4) injected. Isolated pancreata were digested at 37 °C for 8–9 min. After incubation, the digested tissue was washed, hand-shaken and centrifuged at 170 ***g*** for 3 min with HEPES-buffered HBSS (see above) augmented with 5% fetal calf serum (FCS). After passing through a 1 mm tissue-collecting sieve, islets were purified on a discontinuous Ficoll density gradient (Sigma) (Gotoh *et al.* 1985). Islets were hand-picked and transferred into Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% FCS, 11 mM glucose, 100 i.u./ml penicillin and 100 μg/ml streptomycin for further culture.

Ex vivo *gene transfer into mouse islets*

Islets were infected with Ad-eGFP, Ad-AMPK CA or Ad-AMPK DN at a multiplicity of infection (MOI) of 50–100 plaque-forming units per islet cell (assuming islets contained an average of 2000 cells) (Flotte *et al.* 2001) for 16 h. Infection of islets with the null virus at this MOI did not provoke any changes in apoptotic index or glucose-stimulated insulin secretion in comparison to uninfected islets (results herein) (Diraison *et al.* 2004, Parton *et al.* 2004). Islets were then incubated for 48 h at 37 °C in 5% CO_2 prior to *ex vivo* studies, or were transplanted immediately.

Extraction and assay of AMPK activity

For uniformity, mass islet isolations were performed and the resulting islets divided into aliquots for viral infection and comparison. Equal numbers and sized islets were infected with one of each of the three constructs (AdeGFP, AdAMPK-CA or AdAMPK-DN). Quantification of AMPK activity was performed as described previously (da Silva Xavier *et al.* 2003). In brief, 400 transduced islets were scraped into ice-cold lysis buffer (50 mM Tris–HCl, pH 7·4, at 4 °C/250 mM sucrose/50 mM NaF/1 mM sodium pyrophosphate/1 mM EDTA/1 mM EGTA/1 mM DTT/1% (v/v) Triton X-100/complete protease inhibitor mixture; Roche Diagnostics). Extracts were centrifuged (13 000 ***g*** for 5 min at 4 °C), and protein concentration was determined with DC protein assay reagent (Bio-Rad). This protocol releases over 90% of

total cellular content of each AMPK isoform (Salt *et al.* 1998). AMPK was immunoprecipitated from 100 μl cell extract with either sheep anti-α1 or anti-α2 antibodies. AMPK activity was measured in 10 μl crude extract by using 'SAMS' peptide (the synthetic peptide HMRSAMSGLHLVKRR) and γ-^{32}P-ATP (specific activity, 1000 c.p.m./pmol).

Insulin secretion

At 48 h after adenoviral infection, islets were incubated for 60 min at 37 °C in 2 ml Krebs-Ringer bicarbonate HEPES buffer (KRBH) (130 mM NaCl, 3·6 mM KCl, 1·5 mM $CaCl_2$, 0·5 mM $MgSO_4$, 0·5 mM KH_2PO_4, 2·0 mM $NaHCO_3$ and 10 mM HEPES) supplemented with 3 mM glucose and 0·5% (w/v) BSA pre-equilibrated with 95% O_2:5% CO_2, pH 7·4. Islets were separated into three groups of five islets per condition and incubated for 30 min in 1 ml KRBH as above containing either 3 or 17 mM glucose. Total insulin was extracted in acidified ethanol (75% EtOH, 23·5% H_2O and 1·5% HCl). Insulin was measured by radioimmunoassay by competition with ^{125}I-labelled rat insulin (Linco Research, St Charles, MO, USA) according to the manufacturer's instructions. Released insulin is expressed as percentage of the total cellular (extractable) insulin content.

[U-^{14}C]Glucose oxidation

Islets were preincubated for 30 min at 37 °C in KRBH supplemented with 3 mM glucose and 0·5% BSA (w/v). Triplicate groups of 100 islets were then placed in 250 μl KRHB containing 3 or 17 mM unlabelled glucose, 1·7 μCi [U-^{14}C]glucose and 0·5% BSA (w/v) in a 24-well plate. A rubber gasket, the size of the 24-well plate and containing 0·5 cm holes, was aligned over the plate. A CO_2 capture chamber was created as described previously (Collins *et al.* 1998). A UniFilter-24 GF/B plate (Packard Instrument, Research Parkway Meridien, Connecticut, USA) was sealed with an adhesive sheet, and 100 μl of 40% (w/v) KOH were pipetted onto each filter. The filter plate was inverted and aligned over the rubber gasket to form a small CO_2 capture chamber. Finally, the chamber was sealed with a 6 mm glass plate, a 6 mm metal plate and a lead weight to ensure an airtight seal. The apparatus was incubated for 2 h at 37 °C. Filters were removed, and captured $^{14}CO_2$ was measured by scintillation counting. Control incubations lacking islets were included in each incubation series.

Preparation of islet cryostat sections and immunocytochemistry

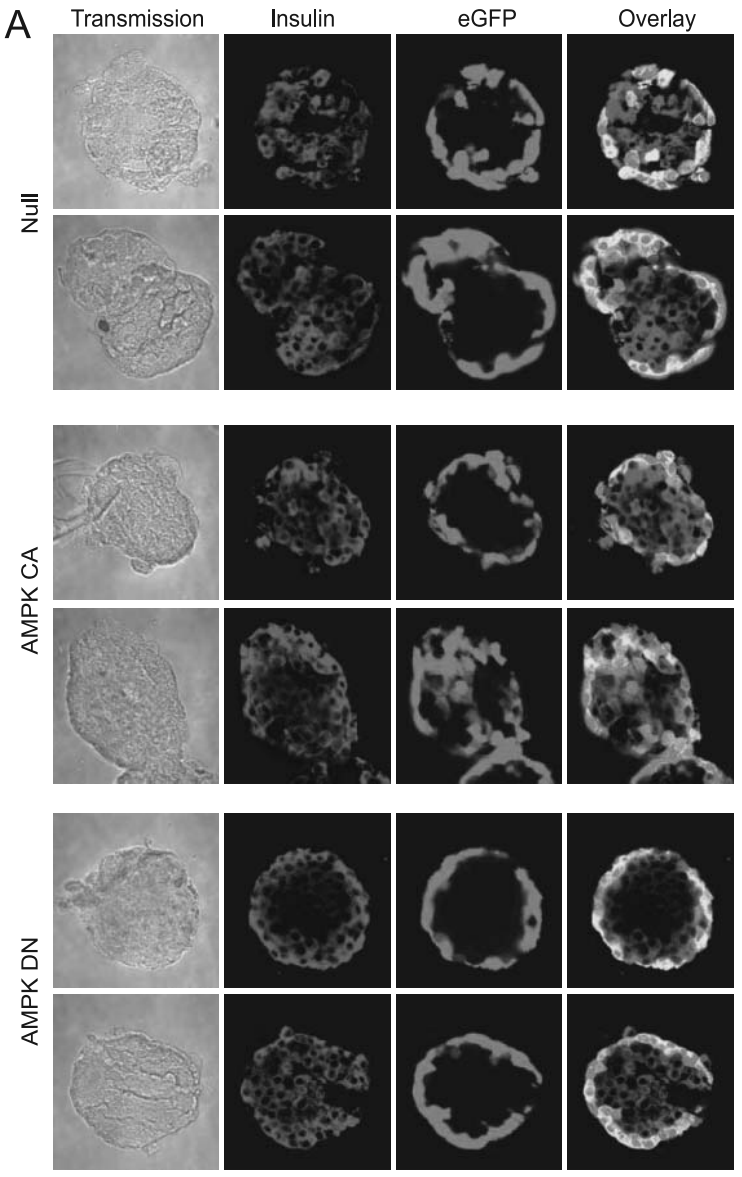
Islets were infected with adenoviruses, cultured for 48 h and then washed with PBS and fixed with 3% (w/v) formaldehyde for 16 h at 4 °C. For sectioning, islets were fixed with Zamboni's fixative (Stefanini *et al.* 1967) overnight at 4 °C, immersed in a solution of 30% (w/v) sucrose in PBS overnight, and subsequently frozen in OCT compound (Tissue-Tek; Sakura Finetecnical, Tokyo, Japan). Islet sections (10 μm) were obtained with a cryostat (Bright OTF5000, Jencons, Leighton Buzzard, UK). Islet slices were permeabilized with 0·3% (w/v) Triton X-100 overnight, and then blocked in 3% (w/v) BSA in PBS for 15 min. Slices were incubated with guinea pig anti-insulin antiserum at 1:500 dilution in a humidified chamber at 4 °C overnight. After washing with PBS, islets were incubated again overnight at 4 °C with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated anti-guinea pig antibody (1:500 dilution). After a final wash in PBS, islet slices were mounted in a mixture of Mowiol (Merck) and glycerol. Images were captured on a Leica SP2 laser scanning confocal microscope with a 63 oil immersion objective with excitation at 350, 488 (Ar) and 543 nm (He-Ne). Emitted light was detected at >515 nm for eGFP (green) or >560 nm for insulin (red).

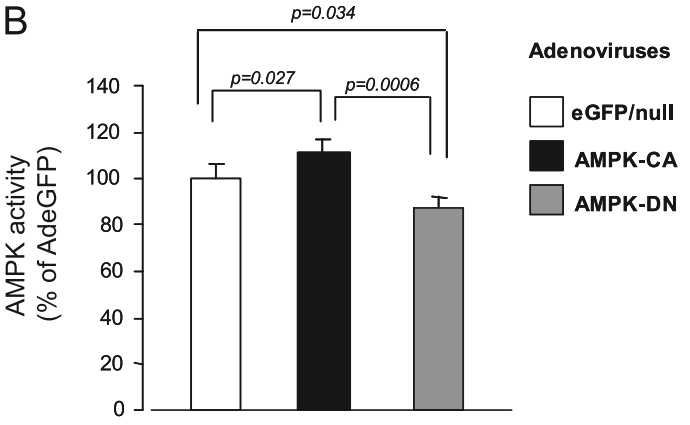
Terminal deoxynucleotide transferase-mediated deoxyuridine trisphosphate nick end-labelling (TUNEL) assay

Islets were cultured for 72 h with null, AMPK CA or DN adenoviruses at 100 MOI as indicated. Islets were then washed twice with ice-cold PBS and 10 μm cryostat sections prepared as above for immunocytochemistry. Sections were permeabilized in 0·2% (v/v) triton X-100 in PBS for 1 h at 4 °C before incubation with TUNEL-labelling solution containing terminal deoxynucleotidyl transfersase (Roche) for 1 h at 37 °C. Slides were then washed twice with PBS and incubated in DAPI (Sigma; 10 μg/ml) in PBS for 10 min. After a final wash in PBS, sections were mounted in movial solution. Positive controls to test the specificity of the TUNEL assay were prepared by incubating in DNase 1 (Roche; 100 U/ml) in 50 mM Tris–HCl, pH 7·5, 1 mg/ml BSA for 10 min at room temperature. Islets were imaged on an SP2 Leica confocal microscope with excitation at 560 nm for TMR-red-labelled nucleotides (red), 488 nm for eGFP (green) and 350 nm for DAPI nuclear staining, and the number of apoptotic cells was counted. The number of TUNEL-positive (apoptotic) cells was counted and expressed as a percentage of total number of cells per islet. At least 12 islets were counted in each case.

Preparation of islets for transplantation

After three gentle washes, transduced islets were hand-picked and carefully counted. Islets were placed into a 1·5 ml tube on ice and pelleted by centrifugation. Supernatant was removed and the islet pellet transferred into a sterile P200 pipette tip plugged with wax. The pipette tip was centrifuged at 170 ***g*** for 3 min, and the supernatant was aspirated to yield a dry islet pellet at its tip.





Islet transplantation

To induce diabetes, animals were fasted overnight and then administered 160 mg/kg of streptozotocin (STZ) (Sigma) in citrate buffer (0·1 M sodium citrate and 0·1 M citric acid, pH 4·5) by an intraperitoneal route. Transplantation was carried out 3 days after administration of STZ. Blood glucose levels were determined by an Accucheck II blood glucose monitor (Roche) using whole blood collected from the tail vein. Mice with a blood glucose value over 20 mmol/l were used as transplant recipients. Mice were anaesthetized by an intraperitoneal injection of fentanyl (10 mg/ml; Hypnorm, Janssen, Wantage, UK) and midazolam (5 mg/ml; Hypnovel) mixed with sterile distilled water in a ratio of 1:1:2, and a volume of 50–100 μl was administered. The left kidney was accessed through a small subcostal incision. A small puncture was made in the renal subcapsule over the inferior pole, and the pipette tip containing the islets was inserted under the capsule and advanced to the superior pole. The islet pellet was then deposited and the pipette tip carefully removed without spillage. The kidney was replaced in the peritoneal cavity and the skin stapled with clips (SLS, Nottingham, UK).

Blood glucose analysis was performed on postoperative day (POD) 1 and thrice weekly to 30 days and thereafter weekly to day 100. In accordance with UK Home Office regulations, animals losing over 15% of preoperative body weight or in distress were killed.

Intraperitoneal glucose tolerance tests

An intraperitoneal glucose tolerance test (IPGTT) was performed on surviving recipients at POD 30. The animals were fasted overnight for not more than 16 h. Each animal was then weighed, and a starved blood glucose value recorded. Animals were fasted overnight, and after administration of a 10% intraperitoneal glucose solution (2 g/kg body mass), samples of blood were taken from the tail vein at 15, 30, 60 and 120 min after the glucose challenge.

Graft nephrectomy

Animals were anaesthetized and the old incision was opened. The left kidney containing the graft was carefully dissected away from peritoneum and adhesions. The renal pedicle was identified and suture ligated with 4/0 vicryl (Ethicon, Edinburgh, UK). The kidney was then excised and the skin closed once more with clips. Blood glucose analysis was performed at 1 and 24 h after graft nephrectomy.

Statistics

Data are given as means ± S.E.M. Comparisons between means were performed with Student's *t*-test for unpaired data, or one-way ANOVA with post hoc Bonferroni correction for measurements of glycaemia, using Graphpad Prism (GraphPad Software Inc., San Diego, CA, USA).

Results

Effects of changes in AMPK activity on glucose metabolism, insulin secretion and apoptosis in isolated mouse islets

Infection of islets with Ad-eGFP, Ad-AMPK CA or Ad-AMPK DN (see Materials and Methods) was confirmed by confocal microscopy to detect eGFP fluorescence. Similar levels of eGFP expression were apparent after infection with each virus (Fig. 1A). The efficiency of infection ranged from ∼25% in the majority of islets (>150 μm) to ∼70% in smaller islets (diameter of <100 μm), with a predominant localization in peripheral, insulin-positive cells as observed previously in rat islets (Diraison *et al.* 2004) (Fig. 1A). Transduction with AMPK-expressing viruses was associated with the expected changes in extractable AMPK activity (Fig. 1B).

Over-expression of AMPK CA in isolated mouse islets inhibited glucose-stimulated insulin secretion (17 versus 3 mM) by approximately 40% ($P<0·01$), but there was no marked effect after expression of dominant-negative AMPK (AMPK DN). Similar findings were observed in rat pancreatic islets where AMPK CA inhibited the secretion of insulin in response to glucose by ∼50% ($P<0·01$) (results not shown) as consistent with previous results (Diraison *et al.* 2003, Leclerc *et al.* 2004). No effect of either virus (CA or DN) on total islet insulin content was observed after infection of islets from either species (data not shown).

We next determined whether the effects of over-expression of AMPK CA may be explained in part by a reduction in glucose oxidation, given the previous finding of a decrease in glucose-induced changes in β-cell ATP content and reduced pyridine nucleotide fluorescence (da Silva Xavier *et al.* 2003). In control mouse islets expressing

Figure 1 Expression of Ad-eGFP, Ad-AMPK CA and Ad-AMPK DN in isolated islets and effect on extractable AMPK activity. (A) Islets from a single isolation were infected with adenoviruses as described (Materials and Methods). Images were captured by confocal scanning microscopy, and overlay projections were constructed. Representative islets are shown. (B) 400 islets per condition then underwent protein extraction and AMPK assay with SAMS peptide (see Materials and Methods). Data are the means ± S.E.M. of three separate experiments.

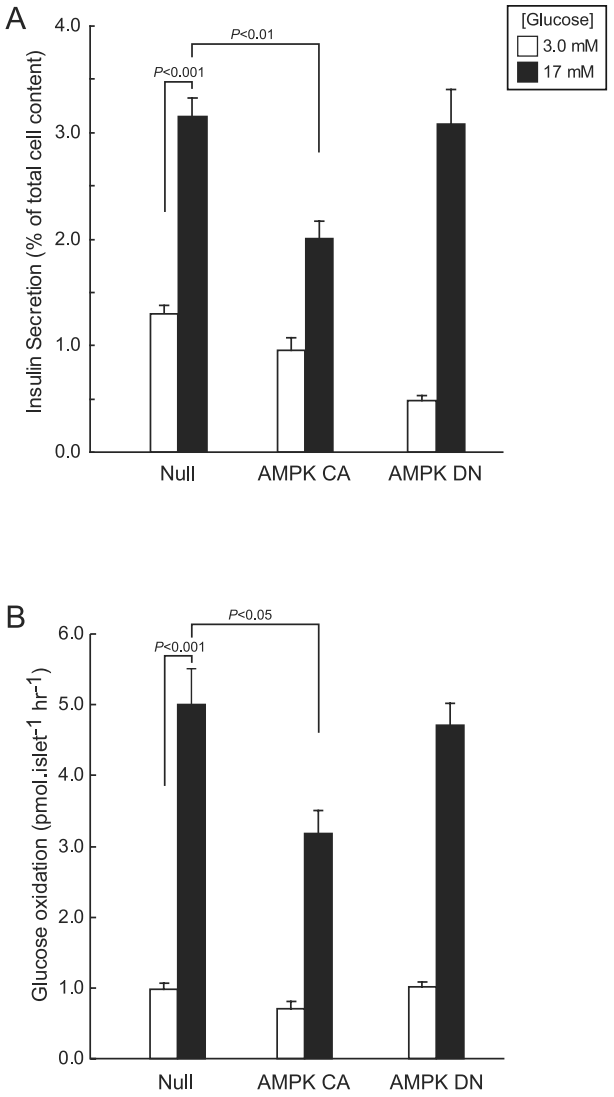


Figure 2 Effect of infection with Ad-eGFP, Ad-AMPK CA or Ad-AMPK DN on glucose-stimulated insulin secretion and glucose oxidation in isolated islets. Islets were infected with the indicated adenoviruses as described (Materials and Methods). (A) After 48 h, insulin secretion was measured during static incubation of islets for 1 h with KRB medium supplemented with indicated glucose concentrations. Total and released insulin were quantified by radioimmunoassay. (B) Glucose oxidation in the three groups of transduced islets was also determined as described (Materials and Methods).

eGFP alone (Null virus-infected), a five-fold induction in the oxidation of [U-^{14}C]glucose was observed at elevated (17 versus 3·0 mM) glucose concentrations (Fig. 2). This increase was significantly (30%, $P<0·05$) inhibited in mouse islets that over-expressed AMPK CA. In contrast, islets transduced with AMPK DN showed no significant difference in the oxidation of glucose at either glucose concentration. Essentially similar data were obtained with rat islets (data not shown).

Table 1 Apoptosis assessed by DNA nick end-labelling (TUNEL) of islet sections. Islets were isolated and cultured for 72 h in adenoviruses as indicated. TUNEL labelling was performed as described in Materials and Methods. The numbers of TUNEL-positive cells are expressed as a percentage of total number of cells per islet. At least 12 islets were counted for each experimental condition

	TUNEL-positive cells (%)
Null (eGFP)	0·68+0·36
AMPK CA	22·8+3·60***
AMPK DN	2·72+1·13
Positive control	76·8+6·37***

***$P<0·001$ versus control adenovirus.

Previous reports have suggested that pharmacological activation of AMPK, or expression of the constitutively activated enzyme in β-cell lines (Kefas *et al.* 2003*a*,*b*) and purified rat β cells (Kefas *et al.* 2003*b*, 2004), can lead to apoptosis. To determine whether over-expression of activated AMPK may induce apoptosis in β cells in the context of the intact, isolated mouse islet, we monitored DNA cleavage in islet slices by incorporating tetramethyl rhodamine-labelled nucleotides into the free 3′-OH end of DNA strand breaks, using terminal deoxynucleotidyl transferase (TUNEL assay). The number of apoptotic cells was then counted in islets infected with null (eGFP), Ad-AMPK CA or Ad-AMPK DN viruses. Whereas expression of AMPK DN had no significant effect on the number of apoptotic cells compared with null virus, AMPK CA caused a ∼30-fold increase after 72 h of infection (Table 1).

Impact of changes in islet graft AMPK activity on glycaemic control

We next explored the cytoprotective and metabolic effects of modulating β-cell AMPK activity *in vivo* by monitoring the survival and function of a suboptimal islet mass transplanted into syngeneic diabetic mice. To this end, a suboptimal islet transplant mass was first determined. Islets were treated with adenoviruses in culture for 16 h before transplantation. This period was chosen to ensure adequate uptake of viral particles by the islet, while avoiding the deleterious effects (e.g. central islet necrosis or changes in islet vasculature) of extended islet culture *in vitro*. Indeed, we observed no recovery of glycaemia after transplantation with islets previously cultured for 48 h, even at high islet numbers (600 islets/animal; $n=8$). We defined a sub-optimal mass as the number of adenovirally infected transplanted islets that would have less than a 25% success rate in restoring euglycaemia (defined as two consecutive post-transplant blood glucose readings below 15 mmol/l). Preliminary data showed that transplantation of 600 islets resulted in a cure rate of 100% ($n=5$), while

transplantation of 300 islets resulted in a cure rate of 25% (n=4). To confirm that the transplantation of 300 islets constituted a suboptimal islet mass, diabetic mice were transplanted with 200 islets, resulting in no improvement in glycaemia compared with untransplanted animals (n=6). These experiments (see Figure 7) established that transplantation of 300 islets constituted a suboptimal, marginal islet mass, likely to be most sensitive to any effect of increasing or decreasing AMPK activity.

To test whether AMPK manipulation could affect β-cell function during the early post-transplantation period, 300 Ad-AMPK CA- or Ad-AMPK DN-infected islets were transplanted into diabetic mice, and blood glucose levels were monitored over a 20-day period. Mice transplanted with 300 Ad-eGFP-infected islets were used as controls. Comparing the mean blood glucose value for mice receiving 300 Ad-AMPK CA islets (n=8) to those mice receiving Ad-eGFP(control) virus-infected islets (n=8) over the 20-day period showed significant impairment in glycaemic control in the Ad-AMPK CA group (P=0·0016; Fig. 3A). By contrast, the mean blood glucose levels for mice receiving Ad-AMPK DN (n=7) islets were not significantly different from those observed in mice receiving Ad-eGFP (control) virus-infected islets (n=8) (P=0·64) (Fig. 3B). However, when mice transplanted with Ad-AMPK CA-infected islets (n=8) and Ad-AMPK DN-infected islets (n=7) (Fig. 3C) were compared, a significant impairment in glycaemic control was seen in the former cohort (P=0·009).

Mice were weighed and monitored regularly over a 30-day postoperative period. All mice transplanted with Ad-eGFP-transduced islets survived this period (n=8). Of the mice receiving Ad-AMPK-CA- or Ad-AMPK-DN-infected islets, 6/8 (75%) and 6/7 (86%) survived respectively. The cohorts' 30-day weight change is shown in Fig. 4. The mean weight change in surviving mice receiving Ad-AMPK CA-infected islets was significantly less than in those mice receiving Ad-AMPK DN-infected islets (–9·0 ± 4·4% vs 3·25 ± 3·25%; P=0·03). There was no significant difference when comparing surviving mice transplanted with Ad-eGFP (control) islets (1·31 ± 4·1%) and either Ad-AMPK CA islets (P=0·06) or Ad-AMPK DN-infected islets (P=0·36).

For a finer assessment of the function of the transplanted islets *in vivo*, an intraperitoneal glucose tolerance test (IPGTT) was performed. The model was established with control non-diabetic mice and STZ-induced diabetic mice without islet transplantation (Fig. 5A). Each contemporaneous cohort of mice underwent IPGTT on POD 30 (Fig. 5A). The mean area under the curve (AUC) in surviving mice receiving Ad-AMPK CA-infected islets (2947 ± 178·6, n=6) was significantly greater than in animals receiving Ad-eGFP-infected islets (2341 ± 183·3, n=8, P<0·04) or Ad-AMPK DN-infected islets (1971 ± 243·8, n=6, P=0·01), demonstrating poorer glucose tolerance (Fig. 5B).

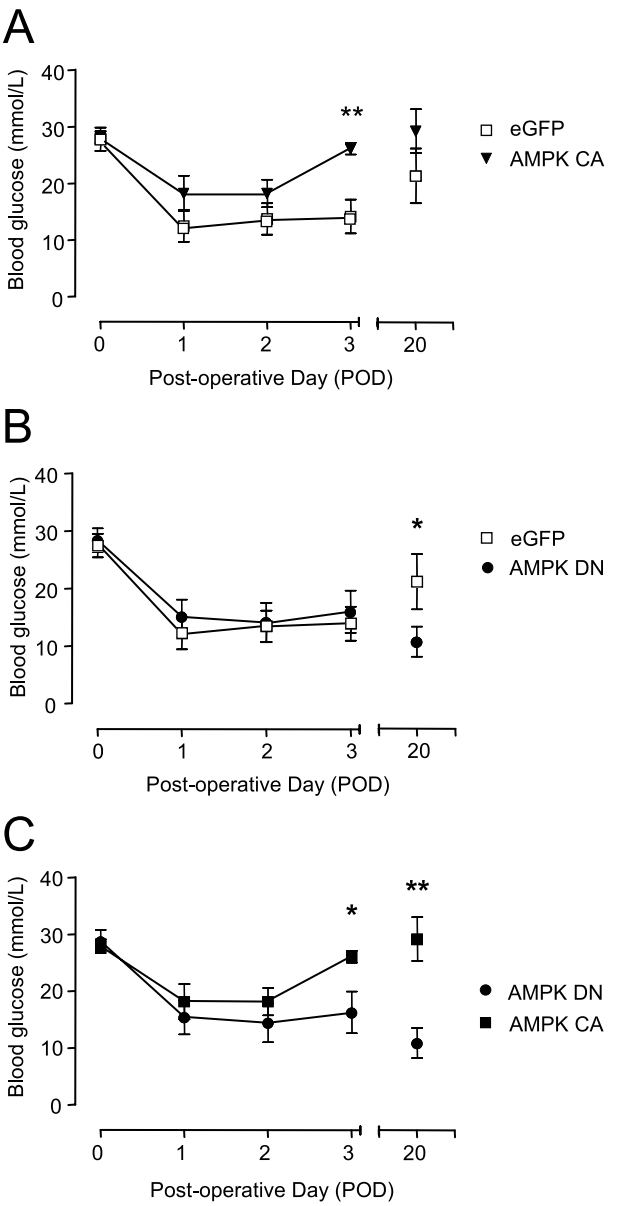


Figure 3 Increased expression of activated AMPK is detrimental to glycaemic control. STZ-diabetic C56BL/6 mice were transplanted with 300 islets infected with Ad-AMPK CA (n=8), Ad-AMPK DN (n=7) or Ad-eGFP/control (n=8) at an MOI of 50–100. Blood glucose analysis was performed preoperatively at day 1 and three times weekly thereafter. (A–C) Mean blood glucose concentration for mice transplanted with Ad-eGFP-, Ad-AMPK CA- or Ad-AMPK DN-infected islets. Data from PODs 4–19 are omitted for clarity. Data are the means ± S.E.M. from the animals shown per condition. **P<0·01 for the effect of Ad-AMPK CA versus Ad-eGFP at POD 3 (A) and versus Ad-AMPK DN at POD 20 (C). *P<0·05 for the effect of Ad-AMPK CA versus Ad-AMPK DN at POD 3 (Unpaired, two-tailed Student's t-test). ANOVA analysis with Bonferroni's multiple comparison test of blood glucose concentration values at all time points in the three animal cohorts confirmed that Ad-AMPK CA-infected islets displayed impaired glycaemic control when compared with either Ad-eGFP- (P<0·01) or Ad-AMPK DN- (P<0·01) transduced islets.

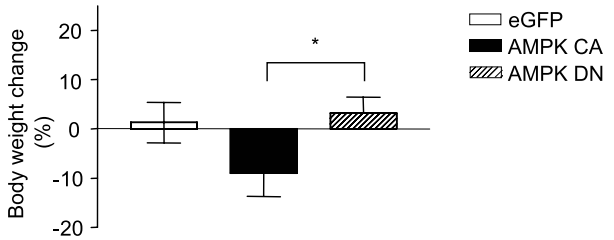


Figure 4 Impact of transplantation with islets infected with Ad-eGFP, Ad-AMPK CA or Ad-AMPK DN on recipient body weight. On postoperative day (POD) 30, surviving mice were weighed, and percentage weight change over the post-transplantation period was compared with the weight immediately before transplantation (POD 0). Data are means ± S.E.M. for the number of animals per condition as follows: Ad-eGFP, $n=8$; Ad-AMPK CA, $n=6$; and Ad-AMPK DN, $n=6$. *$P<0{\cdot}05$ for the difference in weight change in Ad-AMPK CA- and Ad-AMPK DN-transplanted animal cohorts (unpaired Student's *t*-test).

To verify that the changes reported above reflected differences in graft function rather than recovery of endogenous β-cell mass, nephrectomy was performed on a single cohort of three previously transplanted animals, 100 days after the original transplantation. Each animal showed an elevation in blood glucose concentration within 1 h, and at 24 h all were hyperglycaemic (>20 mmol/l) (Fig. 6).

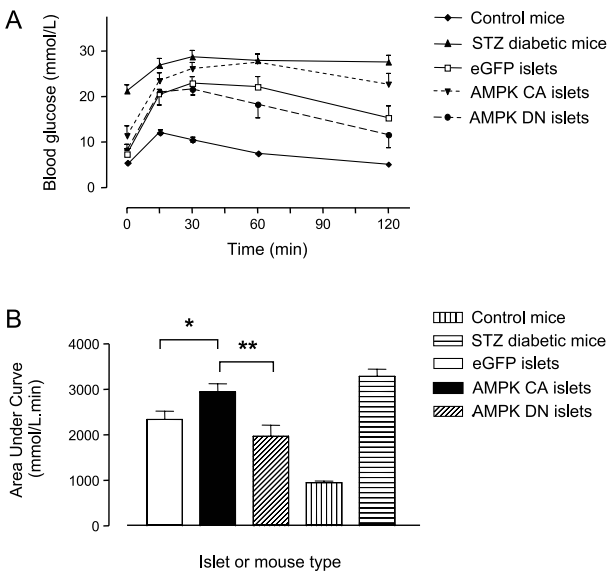


Figure 5 Transplantation of islets transduced with Ad-AMPK CA results in further impairment of glucose tolerance. (A) Intraperitoneal (IP) glucose tolerance tests (IPGTT) were performed in control non-diabetic C57BL/6 mice ($n=8$) and other animals 4 days after STZ administration ($n=7$). After standardized glucose challenge (2 g/kg IP), blood glucose analysis was undertaken. Data were collected for all animals surviving to 30 days. Error bars show mean blood glucose values for each cohort ± S.E.M. (Ad-eGFP, $n=8$ animals; Ad-AMPK CA, $n=6$; Ad-AMPK DN, $n=6$). *$P<0{\cdot}05$ at 60 and 120 min when Ad-AMPK CA- and Ad-AMPK DN-infected islets were compared. (B) Data are mean AUC per condition ± S.E.M. **$P<0{\cdot}01$ for the difference in glucose responsiveness between animals transplanted with Ad-AMPK CA- and Ad-AMPK DN-transduced islets. *$P<0{\cdot}05$ for the difference in glucose responsiveness between animals transplanted with Ad-eGFP- and Ad-AMPK-CA-transduced islets.

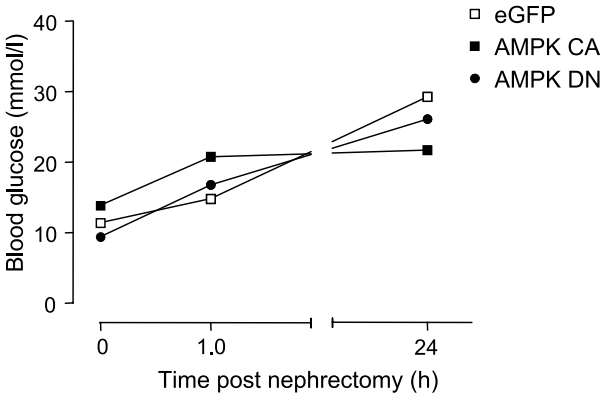


Figure 6 Glycaemic control is dependent upon syngeneic islet graft. A single cohort of three animals (i.e. a single animal per condition) underwent graft nephrectomy, in accordance with UK Home Office regulations. Blood glucose analysis was performed prior to the procedure, and at the indicated times after nephrectomy.

Discussion

Effects of AMP-activated protein kinase on glucose-stimulated insulin secretion in vitro

Over-expression of activated AMPK in intact mouse islets is shown here markedly to suppress glucose oxidation,

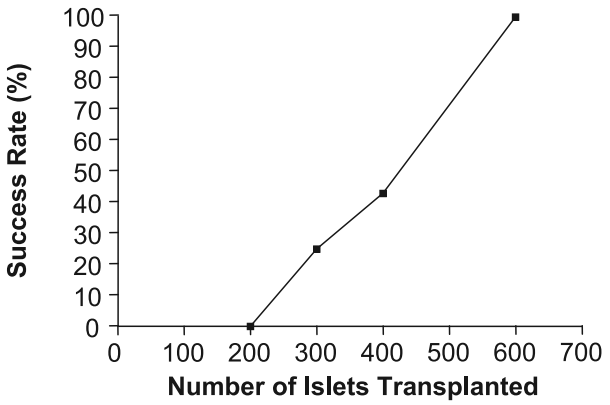


Figure 7 Transplantation of 300 islets represents a suboptimal islet mass. Diabetic mice were transplanted with 600 ($n=5$), 400 ($n=7$), 300 ($n=4$) or 200 ($n=6$) isolated islets that had been incubated for 16 h. All mice transplanted with 600 islets were euglycaemic by POD 3 (blood glucose of<15 mmol/l). Forty-three per cent and 25% of animals transplanted with 400 and 300 islets respectively were euglycaemic by POD 3. None of the animals transplanted with 200 islets achieved euglycaemia over the same time period.

consistent with previous measurements of ATP content and reduced pyridine nucleotide fluorescence in rat islets and clonal β-cells (da Silva Xavier *et al.* 2000, Tsuboi *et al.* 2003). Similarly, glucose-stimulated insulin secretion was inhibited to a similar extent by over-expression of AMPK CA, suggesting that changes in glucose metabolism may be the principal mechanism through which AMPK activation affects glucose-stimulated insulin secretion. Interestingly, these changes were observed despite the relatively low infection efficiency (~20–30%) with the virus, an observation which probably reflects the fact that the outermost layers of β cells are the most active metabolically and in terms of glucose-stimulated insulin release after a period of culture, where degradation of the intraislet vasculature is likely. These levels of infection were not significantly increased in medium-large islets (over ~150 μm diameter) even up to an MOI of ~1000 in our hands, a point at which apoptosis becomes apparent after expression of null (Ad-eGFP) virus (L Parton, F Diraison and G A R, unpublished results; see also Diraison *et al.* 2004).

Expression of AMPK CA also led to a dramatic increase in islet cell apoptosis *in vitro*, a phenomenon which seems likely to contribute substantially to the increased incidence of islet failure after islet transplantation *in vivo*. These observations are consistent with very recent findings showing that activation of AMPK leads to cell-cycle arrest in a number of cell types (Nagata *et al.* 2004, Xiang *et al.* 2004, Jones *et al.* 2005), and with the identification of the tumour suppressor LKB1 as an important upstream AMPK kinase (Hawley *et al.* 2003, Woods *et al.* 2003). Whether apoptosis in β cells is the cause or the consequence of altered glucose metabolism remains to be firmly established, but the fact that treatment of islet or β cells with 5-amino-imidazole carboxamide riboside (AICAR) (da Silva Xavier *et al.* 2003, Leclerc *et al.* 2004) or the AMPK-activator metformin (Leclerc *et al.* 2004) rapidly inhibits insulin release suggests that apoptosis may be a downstream consequence of the metabolic changes (Kefas *et al.* 2004).

By contrast, introduction of AMPK DN was largely without effect on glucose oxidation or insulin release. The latter finding in mouse islets differs from our previous results obtained in rat (da Silva Xavier *et al.* 2000) or human (Leclerc *et al.* 2004) islets, where introduction of AMPK DN caused an increase in insulin release at sub-maximal glucose concentrations. It should be emphasized, however, that no effort was made in the present study to investigate the effects of AMPK selectively on smaller islets, where higher efficiencies of transfection (>70%) were achieved in our earlier studies (Leclerc *et al.* 2004).

Effects of AMPK activation on islet function in vivo

The findings described here extend to the *in vivo* setting the results of the present and previous (da Silva Xavier *et al.* 2000, 2003, Leclerc *et al.* 2004) studies using isolated islets. The decrease in apparent β-cell function observed after the transplantation of islets expressing AMPK CA presumably reflects both the poorer acute response to glucose, and a decrease in β-cell mass due to enhanced apoptosis (see above) (Kefas *et al.* 2003*b*).

A loss of functional β-cell mass of up to 70% occurs after transplantation in man (2). In the present work, activation of AMPK is shown to be detrimental to β-cell function and survival *in vivo*. However, despite a tendency to improved glucose tolerance compared with null virus treatment (Fig. 4A), we were unable to affect islet function significantly after transplantation by expression in islets of a dominant-negative form of AMPK. There may be two possible explanations for this observation. Firstly, endogenous AMPK may remain essentially unstimulated in transplanted islets, at least at early time points. Secondly, the relatively small (~15%; Fig. 1B) decrease in AMPK activity observed *in vitro* seems likely to be due to changes in cells situated at the islet periphery which were efficiently transduced with the virus, leaving cells deeper within the islet core unaffected.

In conclusion, we demonstrate that expression of activated AMPK selectively in pancreatic islets affects glucose metabolism, insulin secretion and β-cell survival, and is associated with decreased β-cell function *in vivo*. We propose that suppression of islet AMPK activity may thus represent a potential therapeutic target for intervention after islet transplantation in type 1 diabetics.

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