Pro-inflammatory cytokines increase glucose, alanine and triacylglycerol utilization but inhibit insulin secretion in a clonal pancreatic β -cell line

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Abstract

We have investigated the effects of prolonged exposure (24 h) to pro-inflammatory cytokines on β -cell metabolism and insulin secretion using clonal BRIN-BD11 β cells. Addition of IL-1 β , tumour necrosis factor- α and IFN- γ (at concentrations that did not induce apoptosis) inhibited chronic (24 h) and acute stimulated levels of insulin release (by 59 and 93% respectively), increased cellular glucose and alanine consumption, and also elevated lactate and glutamate release.

However, ATP levels and cellular triacylglycerol were decreased while glutathione was increased. We conclude that sub-lethal concentrations of pro-inflammatory cytokines appear to shift β -cell metabolism away from a key role in energy generation and stimulus–secretion coupling and towards a catabolic state which may be related to cell defence.

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Introduction

Nutrient metabolism is tightly coupled to insulin secretion in the pancreatic β cell (McClenaghan 2007). Mitochondrial metabolism is crucial for the coupling of glucose and amino acid recognition to exocytosis of insulin granules. This is illustrated by *in vitro* and *in vivo* observations that mitochondrial dysfunction severely impairs insulin secretion. Mitochondria generate ATP which, in addition to Ca^{2+} , is the main coupling messenger in insulin secretion. Mitochondria generate additional coupling factors, which serve as sensors for the control of the exocytotic process (Wollheim 2000, Maechler 2002). Numerous studies have sought to identify the factors that mediate the key amplifying pathway over the Ca^{2+} signal in nutrient-stimulated insulin secretion. Predominantly, these factors are nucleotides (ATP, GTP, cAMP and NADPH), although metabolites have also been proposed, such as long-chain acyl-CoA derivatives and glutamate (Maechler & Wollheim 1999). Glucose, alanine and glutamine metabolism in the β cell may generate many, if not all, of the mitochondrial factors described above (Brennan *et al.* 2002, 2003, Dixon *et al.* 2003, Newsholme *et al.* 2003, Corless *et al.* 2006). Glucose has been reported to protect β cells from apoptosis (Hoorens *et al.* 1996), while glutamine has been reported to afford protection from apoptosis in a wide variety of cells (Curi *et al.* 2005).

We have previously presented ¹³C NMR data which provided novel evidence for substantial intracellular

pancreatic β -cell metabolism of L-glutamine resulting in the formation of L-glutamate, L-aspartate and glutathione (GSH; Brennan *et al.* 2003). We proposed that L-glutamate production is important to the β cell as an intermediate of the γ -glutamyl cycle, which regulates cellular GSH concentration. We have also reported that L-alanine or L-glutamine can chronically regulate gene expression in the clonal β -cell line BRIN-BD11 (Cunningham *et al.* 2005, Corless *et al.* 2006). Differentially regulated genes included the anti-oxidant enzyme catalase and the key regulatory fatty acid synthesis enzyme acetyl-CoA carboxylase (ACC). As both the cell redox status and acyl-CoA derivatives are important for optimal regulation of insulin secretion, chronic amino acid exposure may alter chronic levels of insulin secretion.

Glucose-stimulated insulin secretion was reduced after exposure to the pro-inflammatory cytokines IL-1 β , tumour necrosis factor- α (TNF- α) and IFN- γ in human, mouse and rat islets (Cunningham & Green 1994). Primary pancreatic islets from rats or mice demonstrated reduced glucose oxidation and pyruvate dehydrogenase (PDH) activity after incubation in relatively high concentrations of IL-1 β or TNF- α respectively (Eizirik *et al.* 1988, Park *et al.* 1999). High concentrations of pro-inflammatory cytokines (IL-1 β , TNF- α and IFN- γ) are also known to increase apoptosis in both pancreatic β -cell lines and primary islet cells (Rabinovitch *et al.* 1996). The mechanism by which these pro-inflammatory cytokines induce β -cell apoptosis may be partially dependent on increased inducible nitric oxide

synthase (iNOS) expression and nitric oxide (NO) generation. In a recent paper, it was reported that 46% of the genes modified by cytokines after 8–24 h exposure were NO dependent (Kutlu *et al.* 2003), but inhibition of NO production did not protect INS-1E cells from apoptosis. Similarly in iNOS knockout mice, the rate of apoptosis in primary islets was delayed but not prevented (Liu *et al.* 2000).

Reactive oxygen species (ROS) may contribute to β -cell dysfunction and apoptosis. The source of ROS may be mitochondrial or the plasma membrane associated NADPH oxidase. Expression of a functional NADPH oxidase in islet cells was recently reported, as was up-regulation by IL-1 β or a cocktail of IL-1 β , TNF- α and IFN- γ (Oliveira *et al.* 2003, Morgan *et al.* 2007). Up-regulation of O $_2^{\cdot -}$ generation in the β cell may lead to ROS-dependent damage and also peroxynitrite formation (following the reaction of O $_2^{\cdot -}$ with NO) resulting in subsequent induction of apoptosis. The generation of NO and ROS requires metabolic input, firstly to provide L-arginine, a substrate for NO synthesis, and secondly to provide NADPH, required for both NO and ROS production from iNOS and NADPH oxidase respectively. Indeed, the largest cluster of genes altered on addition of IL-1 β and IFN- γ in primary rat β cells was associated with metabolism (Cardozo *et al.* 2001).

We hypothesized that exposure of β cells to low, non-lethal concentrations of cytokines would initiate a change in metabolism that would favour cell defence at the expense of insulin secretion. Indeed, we now report that chronic (24 h) exposure of clonal BRIN-BD11 β cells to sub-lethal concentrations of IL-1 β , TNF- α and IFN- γ inhibited chronic and acute levels of insulin secretion (by 59 and 93% respectively) but induced significant increases in glucose, alanine and triacylglycerol utilization and glutamate release. The clonal β -cell line BRIN-BD11 was chosen for this work as metabolic, signalling, insulin secretory and cell viability responses to glucose, amino acids as well as other stimuli have been well characterized (McClenaghan *et al.* 1996a, Chapman *et al.* 1999, McClenaghan & Flatt 1999, Brennan *et al.* 2002, 2003).

Materials and Methods

Culture of BRIN-BD11 pancreatic β cells

Clonal insulin-secreting BRIN-BD11 cells were maintained in RPMI-1640 tissue culture medium supplemented with 10% (v/v) fetal calf serum (FCS), 0.1% antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin) and 11.1 mmol/l D-glucose, pH 7.4. The origin of BRIN-BD11 cells are described elsewhere (McClenaghan *et al.* 1996a,b). These cells provide an appropriate β -cell model as evidenced by studies of insulin secretion (McClenaghan *et al.* 1996a,b, McClenaghan & Flatt 1999), β -cell metabolism (Brennan *et al.* 2002, 2003, McClenaghan 2007), electrophysiology (Chapman *et al.* 1999), Ca $^{2+}$ handling (Van Eylen *et al.* 2002) and cellular defence (Conroy *et al.* 2002).

The cells were maintained at 37 °C in a humidified atmosphere of 5% CO $_2$ and 95% air using a Forma Scientific incubator (Marietta, OH, USA). The cells were cultured in 50–70 ml RPMI-1640 tissue culture medium in T175 sterile tissue culture flasks. Cells were subsequently seeded into six-well plates (1.5 $\times$ 10 6 cells per well) and allowed to adhere overnight. Cells were then washed with PBS after which they were incubated in fresh media, containing 11.1 mM D-glucose, 2 mM L-glutamine or other as described below, in the absence or presence of a pro-inflammatory cytokine cocktail (IL-1 β 0.3125 U/ml, TNF α 31.25 U/ml, IFN γ 15.625 U/ml). After 24-h incubation, an aliquot of the media was removed and centrifuged at 200 g for 5 min and used for quantitation of insulin and metabolites (D-glucose, L-glutamine, L-alanine, L-glutamate, TAG and GSH).

Enzymatic determination of metabolites

Glucose The standard glucose concentration in RPMI 1640 medium at the beginning of incubation (11.1 mmol/l in the presence of 2 mmol/l glutamine) and 24 h after culture was determined using a commercially available kit (Sigma). The glucose utilized over the 24-h period was calculated by subtracting the concentration at 24 h from that at 0 h.

L-Alanine The alanine concentration at the beginning of incubation (10 mmol/l added to standard RPMI 1640 containing 11.1 mmol/l glucose and 2 mmol/l glutamine) and 24 h after culture was determined by a spectrophotometric assay based on the oxidation of L-alanine to pyruvic acid and ammonia in the presence of NAD $^+$ and alanine dehydrogenase. The increase in absorbance at 339 nm due to formation of NADH was used to quantify the amount of L-alanine consumed (Dixon *et al.* 2003).

L-Glutamine and L-glutamate The glutamine concentration at the beginning (2 mmol/l in the presence of 11.1 mmol/l glucose) and 24 h after culture was determined based on its hydrolysis to glutamic acid and ammonium ions (NH $_4^+$) in a reaction catalysed by asparaginase. NH $_4^+$ generated combined with 2-oxoglutarate in the presence of NADH to form glutamic acid, NAD $^+$ and water. The concentration of glutamine in the sample was quantified indirectly by measuring the decrease in absorbance at 340 nm due to the conversion of NADH into NAD $^+$. (Dixon *et al.* 2003). The L-glutamate concentration in the culture media was measured using a glutamate dehydrogenase (GDH)-based diagnostic kit (Roche Diagnostics). The L-glutamine is hydrolysed to L-glutamate spontaneously at 37 °C at a rate of approximately 5%/24 h over a concentration range of 1–20 mM. This spontaneous rate of hydrolysis was taken into account when calculating rates of glutamate or NH $_4^+$ production.

Triacylglycerol (triglyceride) Cellular triacylglycerol content was measured as outlined by Dixon *et al.* (2003). Following 24-h culture in the presence or absence of the

cytokine cocktail, cells were washed with PBS, trypsinized and resuspended in 2 mM NaCl, 20 mM EDTA, and 50 mM sodium phosphate (pH 7.4) and sonicated for 1–2 min. Homogenate measuring 10 μ l was mixed with 10 μ l *tert*-butyl alcohol and 5 μ l triton-X-100/methyl alcohol (1:1, v/v) for the extraction of the lipids. Triacylglycerol was determined using a commercial kit (Human, Wiesbaden, Germany) and expressed as μ g/mg protein.

Oxidized and total GSH Oxidized glutathione (GSSG) was quantified in a microtitre plate according to the technique of Baker *et al.* (1990) using the method originally described by Tietze (1969). Cell lysate or GSSG standards (0–500 pmol of GSx/microlitre) were transferred into a microtitre plate and diluted with water. After addition of reaction mixture (0.1 M sodium phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.3 mM DTNB, 0.4 mM NADPH and 1 U/ml GSH reductase, the increase in absorbance at 405 nm was detected at 15-s intervals over a range of 2.5 min using a microtitre plate reader. GSH contents were evaluated using a calibration curve. Intracellular reduced GSH) was measured using the membrane-permeant monochlorobimane (Kamencic *et al.* 2000). Monochlorobimane readily enters the cell where it forms a fluorescent GSH-monochlorobimane adduct that can be measured fluorometrically. This reaction is catalysed by intracellular GSH-S-transferase. This method was used to semi-quantitatively measure the increase or decrease in intracellular GSH after treatment with the cytokine cocktail.

ATP ATP was determined using the luciferase assay (Biovision, Cambs, UK). After 24-h incubation in the presence or absence of the cytokine cocktail, cells were lysed and ATP quantified using the luciferase assay and extrapolated from a standard curve.

Protein determination

Cellular protein was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA kit no. 23225), which utilizes a modification of the biuret reaction.

Western blot analysis

Using RIPA lysis buffer (Upstate Biochemicals, Lake Placid, NY, USA), 20 μ g BRIN-BD11 cell protein extracts were prepared. Samples were subsequently subjected to 6% SDS-PAGE and electrophoretically transferred onto a nitrocellulose sheet. The sheet was blocked in 5% milk protein and incubated with polyclonal anti-ACC, AMPK (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 3% BSA and incubated with polyclonal AMPK-P (adenosine 5'-monophosphate activated kinase-phosphorylated). The blots were washed and probed with horseradish peroxidase and visualized with Supersignal West Pico chemiluminescent substrate (Pierce).

RT-PCR

Total RNA was extracted from BRIN-BD11 cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol applying 50–100 mg of cell extract per 1 mL TRIzol reagent. DNase-treated RNA samples were then used as templates for cDNA synthesis using appropriate primers for LDH (forward primer CCGTTACCTGATGGGAGAAA, reverse primer TTATGCTCTCGCCAAGTCT) or GAPDH as control. The PCR products were resolved on a 1% agarose gel and the intensity of ethidium bromide staining was quantified using the GeneGenius gel documentation and analysis system. Lactate dehydrogenase (LDH) expression changes were calculated relative to the GAPDH expression as a control.

Figure 1 Determination of BRIN-BD11 cellular integrity after 24-h treatment with various concentrations of a pro-inflammatory cytokine cocktail. BRIN-BD11 cells were incubated in RPMI-1640 media supplemented with 10% (v/v) FCS and 2 mmol/l glutamine. Cell integrity (%) was measured by either LDH release compared with total LDH concentrations (A), determination of mitochondrial viability using the MTT assay (B) or determining the level of apoptosis and necrosis (C) using a DNA fragmentation assay (Roche Diagnostics). Cytokine levels used in cell incubations were dilutions from a stock of concentration; 1 $\times$ = IL-1 β (10 ng/ml), TNF- α (7.5 ng/ml), IFN- γ (500 ng/ml), which is equivalent to IL-1 β (5 U/ml), TNF- α (500 U/ml), IFN- γ (250 U/ml). Results are expressed as mean $\pm$ s.d. * P < 0.05, ** P < 0.01.

Figure 2 The effect of pro-inflammatory cytokines on glucose, glutamine and alanine consumption and lactate, glutamate and glutathione production in BRIN-BD11 cells. Cells were incubated in RPMI-1640 media supplemented with 10% (v/v) FCS and 2 mmol/l glutamine and 10 mmol/l alanine where indicated. Glucose (A), alanine (B) and glutamine (C) consumption after 24-h treatment of BRIN-BD11 cells with the pro-inflammatory cytokine cocktail. Lactate (D), glutamate (E) and glutathione (F) production after 24-h treatment of BRIN-BD11 cells with the pro-inflammatory cytokine cocktail. Results are $n=6$ in duplicate (A, C–E), $n=3$ in duplicate (B), $n=3$ (F). * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Chronic (24 h) and acute levels of insulin secretion

BRIN-BD11 cells were seeded in 24-well plates (1×10^5 cells/well) in RPMI-1640 medium containing 11.1 mM D-glucose in the absence or presence of the cytokine cocktail (IL-1 β 0.3125 U/ml, TNF α 31.25 U/ml, IFN γ

15.625 U/ml). After 24-h incubation, an aliquot of the media was removed and centrifuged at 200 g for 5 min and analysed for insulin content using the Mercodia Ultrasensitive Rat Insulin ELISA kit (Uppsala, Sweden). Additionally, some cells were further incubated for 40 min in the presence of 1.1 mmol/l glucose followed by 20 min in the presence of 16.7 mM glucose

Figure 3 The effect of pro-inflammatory cytokines on BRIN-BD11 β -cell lactate dehydrogenase expression. RT-PCR analysis of LDH expression levels in BRIN-BD11 cells incubated for 24 h in the absence or presence of the pro-inflammatory cytokine cocktail. RNA extraction and analysis was as described in the Materials and Methods section. The PCR products were resolved on a 1% agarose gel (A) and the intensity of ethidium bromide staining was quantified using the GeneGenius gel documentation and analysis system before displaying relative to GAPDH (B).

and 10 mM alanine (a stimulus that results in a robust and reproducible secretory response in normal conditions, McClenaghan *et al.* 1996a,b, Brennan *et al.* 2002), when an aliquot of the incubation medium was removed, centrifuged at 200 *g* for 5 min and analysed for insulin using the Mercodia Ultrasensitive Rat Insulin ELISA kit. The insulin content of cells was not significantly different in any of the incubation conditions described in this paper.

Statistical analysis

The results are presented as mean $\pm$ S.D. Groups of data were compared using a Student's unpaired *t*-test or ANOVA where appropriate. Differences were considered significant at a *P* value of <0.05 .

Results

Pro-inflammatory cytokine-induced changes in cellular integrity

Cell integrity was investigated by use of three different methods. LDH release can be determined after loss of plasma membrane integrity. This assay demonstrated that there was no significant change in % cell death after 24-h treatment with the pro-inflammatory cytokine cocktail up to and including a concentration of $1/16\times$ ($1\times$ consisted of IL- 1β 5 U/ml, TNF α 500 U/ml, IFN γ 250 U/ml). At $1/8\times$ there was an increase from 9.9 ± 4.7 to $16.3 \pm 4.2\%$ $P=0.057$. At higher concentrations of $1/4\times$ and $1/2\times$, there were further losses in cellular plasma membrane integrity (Fig. 1A). Examination of mitochondrial viability using the MTT assay demonstrated that there was no significant change in activity after treatment with the pro-inflammatory cytokine cocktail up to and including $1/2\times$ (with respect to IL- 1β 5 U/ml, TNF α 500 U/ml, IFN γ 250 U/ml). This result would indicate that BRIN-BD11 cell mitochondria are relatively resistant to cytokine-induced damage.

Assessment of apoptotic cell death by determination of DNA fragmentation, based on an ELISA, demonstrated that there was no significant change in apoptotic cell death after 24-h treatment with the pro-inflammatory cytokine cocktail, up to and including $1/16\times$ (where $1\times$ = IL- 1β 5 U/ml, TNF α 500 U/ml, IFN γ 250 U/ml). A fourfold increase in fragmentation was seen after treatment with $1/8\times$ which further increased to a sevenfold increase after treatment with $1/2\times$ (Fig. 1C). Examination of necrotic cell death using the DNA ELISA revealed that there was no significant increase in necrotic death until a cocktail concentration of $1/4\times$ was used, which resulted in a fourfold increase in necrosis which further increased to a fivefold increase after treatment with a concentration of $1/2\times$ (Fig. 1C).

Pro-inflammatory cytokine induced-changes in glucose, alanine and glutamine consumption

The concentration of the cytokine cocktail chosen for further experiments was $1/16\times$ (corresponding to final incubation concentrations of IL- 1β 0.3125 U/ml, TNF α 31.25 U/ml and IFN γ 15.625 U/ml) as this concentration did not result in significant changes in cellular integrity. There was a twofold significant increase in glucose consumption after treatment with the pro-inflammatory cytokine cocktail (20.47 ± 5.31 to 44.61 ± 6.67 μ mol glucose/mg protein/24 h, $P<0.001$; Fig. 2A).

Alanine consumption was also significantly increased after treatment with the pro-inflammatory cytokine cocktail (4.38 ± 2.31 to 10.92 ± 1.90 μ mol alanine/mg protein/24 h, $P<0.01$), corresponding to a 150% increase in alanine consumption over the 24-h period (Fig. 2B). In contrast,

Figure 4 The effect of pro-inflammatory cytokines on ATP levels and insulin secretion in BRIN-BD11 cells. Exposure to a pro-inflammatory cytokine cocktail for 24 h changed intracellular ATP concentration (A), chronic levels of insulin secretion (B) and acute stimulation of insulin secretion using a stimulus of 16.7 mM glucose plus 10 mM alanine (C) from BRIN-BD11 cells. Results are expressed as mean $\pm$ S.D. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with incubation in the absence of cytokines.

Figure 5 The effect of pro-inflammatory cytokines on ACC, AMPK and intracellular triacylglycerol levels in BRIN-BD11 cells. Protein expression of ACC1, AMPK and phosphorylated AMPK in BRIN-BD11 cells were determined by western blot analysis (Panels A, B, C respectively) after 24-h treatment with the pro-inflammatory cytokine cocktail. Results are expressed as mean $\pm$ s.d. using GAPDH as an expression control (Panel D) * $P < 0.05$. Intracellular triglyceride concentration of BRIN-BD11 cells after 24-h treatment with the pro-inflammatory cytokine cocktail (Panel E). Results are expressed as mean $\pm$ s.d. for $n = 3$ in duplicate. ** $P < 0.01$.

glutamine consumption was significantly decreased by the pro-inflammatory cytokine cocktail (9.90 ± 0.49 to 7.00 ± 0.21 μmol glutamine/mg protein/24 h, $P < 0.01$), corresponding to a 29% reduction in glutamine consumption over the 24-h period (Fig. 2C).

Pro-inflammatory cytokine-induced changes in lactate, glutamate and GSH production

A 271% increase in lactate production was observed after treatment with the pro-inflammatory cytokine cocktail. After 24-h incubation, lactate production had increased from 15.87 ± 6.09 to 58.81 ± 13.11 μmol lactate/mg protein, $P < 0.001$ (Fig. 2D). LDH expression and activity were determined after 24-h incubation in the absence or presence of the pro-inflammatory cytokine cocktail. The LDH expression was not altered after exposure to the pro-inflammatory cytokine cocktail (Fig. 3A and B) and neither was V_{max} activity determined via kinetic plot (V_{max} after incubation in the absence or presence of the pro-inflammatory cytokines for 24 h was 453 ± 52 or 579 ± 124 nmol/mg protein/min respectively). Glutamate release significantly increased from 0.87 ± 0.28 to 1.30 ± 0.28 μmol glutamate/mg protein after 24 h, a 49% increase in glutamate release, $P < 0.05$ (Fig. 2E). Intracellular levels of reduced GSH, significantly increased by 1.6-fold after 24 h, $P < 0.001$ (Fig. 2F). Similarly intracellular levels of GSSG, were increased by 1.4-fold after treatment with the pro-inflammatory cytokine cocktail for 24 h, $P = 0.07$.

Pro-inflammatory cytokine-induced changes in ATP concentration and insulin secretion

ATP concentration was determined by luciferase assay after treatment for 24 h in the presence of various concentrations of the pro-inflammatory cytokine cocktail. There was a 20% reduction in intracellular ATP concentration (from 47.56 ± 9.20 to 38.04 ± 4.14 pmol ATP per mg protein, $P < 0.01$, Fig. 4A). The fall in ATP content after 24 h may reflect increased rates of consumption of ATP relative to generation.

A 59% reduction in chronic insulin secretion was observed after 24-h treatment of BRIN-BD11 cells with the pro-inflammatory cytokine cocktail. Total insulin secreted over the 24-h period was reduced from 39 ± 7 to 16 ± 3 μg insulin/mg protein/24 h, $P < 0.001$ (Fig. 4B). A more dramatic inhibition of an acute (40 min) nutrient stimulus (16.7 mM glucose plus 10 mM alanine) of insulin secretion was observed after 24-h treatment of BRIN-BD11 cells with the pro-inflammatory cytokine cocktail. A 93% reduction in stimulated insulin secretion (over basal) was observed (Fig. 4C).

Pro-inflammatory cytokine-induced changes in ACC, AMPK, AMPK-P protein expression and triacylglycerol levels

Fatty acid synthesis is essential for acute and chronic stimulation of insulin secretion (see Newsholme *et al.* 2007 for review). Fatty acid synthesis is regulated by the enzyme ACC which itself is covalently modified by phosphorylation by the AMP-regulated kinase (AMPK). Phosphorylation of ACC by AMPK will reduce activity. No change in the

Figure 6 The effect of cytochalasin D on chronic glutamate release from BRIN BD11 cells. Glutamate production (A) and chronic insulin secretion (B) after 24-h treatment of BRIN BD11 cells in RPMI-1640 media supplemented with 10% (v/v) FCS and 2 mmol/l glutamine in the absence or presence of 1 mM cytochalasin D. Results are expressed as mean $\pm$ s.d. * $P < 0.05$, ** $P < 0.01$.

expression of the key fatty acid synthesis enzyme ACC was observed after 24-h incubation in the presence of the pro-inflammatory cytokine cocktail, $P = 0.63$ (Fig. 5A).

ACC activity is negatively regulated by phosphorylation by the key signal-transducing kinase, AMPK. A slight decrease in AMPK levels was observed after 24-h exposure to the pro-inflammatory cytokine cocktail, but this was not significant. Results were normalized and expression of AMPK was reduced from an arbitrary value of 1 to 0.73 ± 0.32 units, $P = 0.23$ (Fig. 5B).

However, a significant increase in phosphorylated AMPK (therefore activated AMPK) levels was observed after 24-h treatment with the cytokine cocktail. Results were normalized and expression of AMPK-P increased from 1 ± 0.59 to 1.75 ± 0.45 units, $P = 0.03$ (Fig. 5C), which may indicate higher levels of phosphorylated ACC, resulting in inhibition of fatty acid synthesis and promotion of FA oxidation.

In support of the latter finding, intracellular triacylglycerol levels were reduced from 0.12 ± 0.02 to 0.06 ± 0.04 mg triglyceride per mg of protein after 24-h incubation in the presence of cytokines, a reduction of 49%, $P < 0.01$ (Fig. 5D). Triacylglycerol synthesis is thought to be cytoprotective in the context of lipotoxicity, but hydrolysis may contribute to stimulation of insulin secretion under appropriate conditions (see Newsholme *et al.* 2007 for review).

Stimulation of glutamate release by cytochalasin D (CCD)

There was a significant increase in glutamate export after treatment with the actin microfilament disruptor CCD

(1 μ M). Glutamate export increased from 1.026 ± 0.23 to 2.92 ± 1.72 μ mol glutamate/mg protein/24 h, $P = 0.04$, a 185% increase after treatment with 1 μ M CCD (Fig. 6A). There was a significant reduction in chronic insulin secretion after 24-h treatment with 1 μ M CCD. Insulin secretion was reduced from 39.64 ± 6.85 to 14.17 ± 6.33 μ g insulin/mg protein/24 h treatment in the presence of 1 μ M CCD, a 64% reduction in chronic insulin secretion $P < 0.001$ (Fig. 6B).

Discussion

Previous studies have reported that exposure of rat or mouse pancreatic islets to relatively high concentrations of the pro-inflammatory cytokines IL-1 β or TNF- α or a mixture of IL-1 β , TNF- α and IFN- γ resulted in decreased glucose oxidation, decreased activity of glycolytic enzymes and inhibition of insulin secretion (Eizirik *et al.* 1988, Park *et al.* 1999, Wallstrom *et al.* 2003). However, relatively high concentrations of cytokines were previously used (for example, 25 U/ml IL-1 β 1000 U/ml TNF- α and 1000 U/ml IFN- γ , Wallstrom *et al.* 2003), which resulted in rapid stimulation of apoptosis. The study described here employed considerably lower concentrations of pro-inflammatory cytokines (80-fold, 32-fold and 64-fold for IL-1 β , TNF- α and IFN- γ respectively, IL-1 β 0.3125 U/ml, TNF- α 31.25 U/ml, IFN- γ 15.625 U/ml) and found enhanced glucose, alanine and triacylglycerol utilization together with concomitant increases in lactate, glutamate and GSH production. The effect of low concentrations of pro-inflammatory cytokines (equivalent to those used in this study) in primary islets has not been addressed. With respect to enzyme activity measurement, we found that maximal LDH activity was not altered after incubation in the presence of pro-inflammatory cytokines. However, both chronic and acute levels of insulin secretion were substantially reduced in agreement with previous reports using higher concentrations of cytokines (Cunningham & Green 1994, Cunningham *et al.* 2005). At the cytokine concentrations used in this study, no change was observed in LDH release, MTT reduction or DNA fragmentation after 24 h thus indicating cellular integrity was maintained under these conditions. Thus, a major question concerns the role of increased fuel utilization in β cells exposed to sub-lethal concentrations of pro-inflammatory cytokines. The additional glucose consumed appeared to be mainly converted to lactate, thus suggesting a shift to anaerobic glycolysis and a shift away from glucose-dependent stimulation-secretion coupling. Partial similarity thus exists with *in vivo* studies demonstrating partial pancreatectomy-induced hyperglycaemia resulted in up-regulation of rat islet cell LDH and monocarboxylate (lactate) transporter gene expression (Laybutt *et al.* 2002) and thus desensitization to glucose. Cytokines have been shown to increase NO production, NADPH oxidase expression and ROS production ((Eizirik & Darville 2001, Cunningham *et al.* 2005, Morgan *et al.* 2007). The NADPH oxidase complex uses molecular oxygen and NADPH (as a source of reducing

equivalents) to generate O_2^- . The source of NADPH is critically important to the β cell, which has a relatively low pentose phosphate pathway activity (Papaccio *et al.* 2005). Interestingly, the β cell also contains malic enzyme (NADP⁺ - dependent malate dehydrogenase), capable of converting malate to pyruvate with the concomitant production of NADPH from NADP⁺ (MacDonald 1995). The function of NADPH in the β cell, although positively correlated with insulin secretion, has

never been fully clarified but it may contribute to anti-oxidant mechanisms including the GSH reductase/GSH peroxidase system. We would suggest that NADPH may additionally contribute to ROS generation via its role as substrate in the NADPH-oxidase-catalysed reduction of molecular oxygen. While the full role of ROS in the β cell is uncertain, it may influence redox-sensitive enzymes, signal transduction components or transcription factors, which determine insulin

Figure 7 Glucose and amino acid metabolism in cytokine challenged clonal pancreatic β -cells. Metabolic scheme outlining the increase (+) or decrease (–) in utilization or production of various metabolites in the β -cell line BRIN BD11 in response to exposure to a sub-lethal concentration of IL-1 β , TNF- α and IFN- γ .

gene transcription or insulin secretion or both. At the low concentration of pro-inflammatory cytokines used in the present study, we did not find a change in expression of the NADPH oxidase subunit p47^{phox} (results not shown). However, under adverse conditions such as sustained exposure to higher concentrations of pro-inflammatory cytokines, the resulting up-regulation and activity of NADPH oxidase may result in excessive ROS initially causing oxidative stress, increased glucose consumption and lactate production but reduced ATP production. However, enhanced ATP consumption resultant from initiation of cell defence mechanisms including activation of ion pumps and transporters will result in a critical fall in ATP levels, insulin secretion and finally apoptosis (Brownlee 2003). That the pro-inflammatory cytokine cocktail decreased β -cell triacylglycerol level was unexpected, indicating a shift towards fatty acid oxidation or export (Martins *et al.* 2004). One possibility that might explain the functional impact of TAG hydrolysis in β cells is enhanced TAG/FA cycling stimulated by pro-inflammatory cytokine exposure. This can be expected to consume considerable amounts of glucose (as a supplier of glycerol 3-phosphate for esterification) and ATP. The diversion of ATP to TAG/FA cycling and release of potentially high intracellular concentrations of NEFA may negatively impact on β -cell function and viability (Nolan *et al.* 2006). The observed increase in phosphorylated AMPK would be expected to enhance ACC phosphorylation and inhibition, correlating well with the cytokine-induced shift towards fatty acid oxidation. The loss of fatty acid synthesis and associated lipid signalling molecule generation via this pathway (Haber *et al.* 2006, Newsholme *et al.* 2007) could also contribute to the observed decrease in insulin secretion.

Chronic glutamate release from the β cell has been reported (Corless *et al.* 2006) and suggested to play a role in autocrine/paracrine inhibition of insulin secretion via activation of metabotropic or ionotropic glutamate receptors described in pancreatic β cells and islets (Molnar *et al.* 1995). The present results demonstrate that pro-inflammatory cytokines increase glutamate release, which might be relevant in the observed inhibition of secretion via glutamate receptor activation. Furthermore, cytochalasin D, an actin microfilament disruptor (Head *et al.* 2006) enhanced glutamate release from BRIN-BD11 cells. Since microfilaments play an important role in the movement of insulin granules to the plasma membrane, glutamate may be important for vesicle transport towards exocytotic sites. Disruption of granule movement and fusion might also decrease the requirement for glutamate which was subsequently exported. Interestingly, pro-inflammatory cytokines might also enhance glutamate formation from precursors such as glucose (Brennan *et al.* 2003, Broca *et al.* 2003) or alanine (Dixon *et al.* 2003). This might explain the observed inhibition of glutamine utilization by cytokines.

From the above considerations, pro-inflammatory cytokines appear to shift β -cell metabolism away from stimulus-secretion coupling and towards a catabolic state related to cell defence (see Fig. 7). The endoplasmic reticulum is essential for normal protein synthesis and folding and is crucial for

cellular integrity and responses to stress. Cytokines provoke alterations in normal ER function and environment leading to accumulation of unfolded proteins and activation of a specific ER stress response, also known as the unfolded protein response (Cardozo *et al.* 2005). Three ER signal transduction pathways appear to be important for this stress response. Subsequent downstream events associated with ER-directed transcriptional activation and protein synthesis will place a considerable energy burden on the β cell.

In contrast to the reduction in glucose transport and metabolism in muscle and adipose tissues, inflammatory cytokines have been reported to stimulate glucose uptake and metabolism in both T and B lymphocytes (Frauwirth & Thompson 2004, Doughty *et al.* 2006). Of course, cytokine stimulation of lymphocyte growth, maturation and proliferation is well defined and is required for an appropriate immune response. We conclude that enhanced glucose metabolism in response to non-lethal concentrations of cytokines appears to be related to enhanced NADPH production, enhanced metabolic intermediate generation for biosynthetic pathways and enhanced energy requirements. Pro-inflammatory cytokines do not appear to stimulate β -cell proliferation *in vitro* or *in vivo*. However, enhanced metabolic intermediate generation may be required for β -cell protection and survival. The elevation in GSH levels in β cells observed in this study would be consistent with activation in cellular protective mechanisms. Further studies are required to explore these possibilities and ultimately their application to primary human β cells.

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