

Chronic exposure to leucine *in vitro* induces β -cell dysfunction in INS-1E cells and mouse islets

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Abstract

Chronic hyperglycemia and hyperlipidemia cause deleterious effects on β -cell function. Interestingly, increased circulating amino acid (AA) levels are also a characteristic of the prediabetic and diabetic state. The chronic effects of AAs on β -cell function remain to be determined. Isolated mouse islets and INS-1E cells were incubated with or without excess leucine. After 72 h, leucine increased basal insulin secretion and impaired glucose-stimulated insulin secretion in both mouse islets and INS-1E cells, corroborating the existence of aminoacidotoxicity-induced β -cell dysfunction. This took place concomitantly with alterations in proteins and genes involved in insulin granule transport, trafficking (e.g. collapsin response mediator protein 2 and GTP-binding nuclear protein Ran), insulin signal transduction (proteasome

subunit α type 6), and the oxidative phosphorylation pathway (cytochrome *c* oxidase). Leucine downregulated insulin 1 gene expression but upregulated pancreas duodenum homeobox 1 and insulin 2 mRNA expressions. Importantly, cholesterol (CH) accumulated in INS-1E cells concomitantly with upregulation of enzymes involved in CH biosynthesis (e.g. 3-hydroxy-3-methylglutaryl-CoA reductase, mevalonate (diphospho) decarboxylase, and squalene epoxidase) and LDL receptor, whereas triglyceride content was decreased. Our findings indicate that chronic exposure to elevated levels of leucine may have detrimental effects on both β -cell function and insulin sensitivity. Aminoacidotoxicity may play a pathogenic role in the development of type 2 diabetes.

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Introduction

Type 2 diabetes (T2D) involves both defective insulin secretion and insulin resistance (IR). In T2D, hyperglycemia and hyperlipidemia exert deleterious effects on β -cell function, referred to as glucotoxicity and lipotoxicity (Xiao *et al.* 2001, El-Assaad *et al.* 2010), with increased basal insulin secretion (BIS) and impaired glucose-stimulated insulin secretion (GSIS). Patients with impaired glucose tolerance (IGT) or in the early stage of T2D present with characteristic β -cell dysfunction (Ferrannini *et al.* 2003), but the nature of the primary β -cell defect is still elusive.

In an IR state, plasma concentrations of amino acids (AAs) are elevated, particularly leucine, isoleucine, valine, proline, and glutamine (Newgard *et al.* 2009). Thus, elevated levels of certain AAs are also a characteristic of the diabetic state and may play a pathogenic role in the development of T2D in addition to hyperglycemia and dyslipidemia.

Most AAs, including leucine, acutely stimulate insulin secretion from pancreatic β cells in a dose- and glucose-dependent manner (Liu *et al.* 2008), and leucine stimulates insulin secretion by serving as both a metabolic fuel and an allosteric activator of glutamate dehydrogenase

(Newsholme *et al.* 2006, MacDonald *et al.* 2008). Leucine and its transaminated product α -ketoisocaproate may also affect insulin secretion via direct inhibition of β -cell ATP-regulated potassium channel currents (Newsholme *et al.* 2006). Correspondingly, acute *in vivo* studies consistently revealed that additional leucine intake increased insulin secretion and reduced postprandial blood glucose (Kalogeropoulou *et al.* 2008). However, there are conflicting results on the long-term effects of leucine on β -cell function. Long-term exposure of pancreatic islets to high leucine levels has been shown to selectively impair GSIS, which is associated with a reduced ATP-to-ADP ratio (Anello *et al.* 2001). Moreover, Zhang *et al.* (2009) reported that chronic exposure to leucine downregulates the expression of pancreas duodenum homeobox 1 (*Pdx1*), glucokinase (*Gk* (*Gck*)), and glucose transporter type 2 in rat insulinoma β cells, resulting in decreased insulin content and GSIS at high glucose. However, this was challenged by the observation that leucine upregulates the ATP synthase β subunit (*ATP β*) mRNA level in RIN5mf cells and increases GSIS, ATP, GCK, and ATP β levels in rat and human islets (Yang *et al.* 2006). Conflicting results were also observed when rats were fed leucine orally for varying number of weeks. Mice given a high-fat diet with doubled

dietary leucine intake were significantly more glucose tolerant and insulin sensitive than high-fat diet mice without additional leucine (Zhang *et al.* 2007). By contrast, Balage *et al.* (2011) observed that leucine-supplemented rats displayed IGT and had a significant increase in visceral adipose tissue.

Given such conflicting results on the actions of leucine, we wanted to elucidate the long-term actions of leucine on β -cell function *in vitro*. Here, we show that chronic exposure to excess leucine induces characteristic diabetic changes in β -cell function (i.e. increases BIS and hampers GSIS) and alters β -cell gene and protein expressions. We found that leucine-induced β -cell dysfunction is related to abnormal cholesterol (CH) metabolism with corresponding accumulation of CH content, impaired oxidative phosphorylation (OxPhos), impaired insulin secretory granules (ISGs) transport, and trafficking.

Materials and Methods

Reagents and buffers/solutions

All chemicals were from Sigma–Aldrich if not stated otherwise. Modified Krebs–Ringer buffer (M-KRB) contained (mM) NaCl, 125; KCl, 5.9; MgCl₂, 1.2; CaCl₂, 1.28; NaHCO₃, 5.0; and HEPES, 25; pH 7.4. SYTO 24 solution: 5 mM SYTO 24 green fluorescent nucleic acid stain (Molecular Probes, Invitrogen, Eugene, OR, USA) dissolved in DMSO ($\geq 99.9\%$) to a final concentration of 0.01 mM. Glycine–BSA buffer (pH 8.8) contained glycine 100 mM and 0.25% BSA. 2D-lysis buffer contained 6 M urea, 2 M thiourea, 1.5% (w/v) pharmalyte, 0.8% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, and 1% (w/v) dithioerythritol in water. The rehydration buffer consisted of the same substances, in the same concentrations as the lysis buffer, but with extra pharmalyte (5 μ l/ml).

Animals

Adult female NMRI mice (Bomholtgaard Breeding and Research Centre, Ry, Denmark) weighing 20–25 g were used. The animals were kept on a standard pellet diet and tap water *ad libitum* and a 12 h light:12 h darkness cycle. The study was carried out in accordance with the guidelines of the Danish Council for Animal Experiments.

Islet isolation

Islets were isolated by the collagenase digestion technique (Lacy & Kostianovsky 1967). In brief, the animals were anesthetized *i.p.* with pentobarbital sodium (50 mg/kg), and a midline laparotomy was performed. The pancreas was retrogradely filled with 3 ml ice-cold Hanks Balanced Salt Solution (HBSS) supplemented with 0.3 mg/ml

collagenase P (Boehringer Mannheim). The pancreas was removed, incubated for 19 min at 37 °C in a water bath, and subsequently rinsed with ice-cold HBSS, after which the islets were handpicked under a stereomicroscope. The islets were incubated overnight at 37 °C and 95% normal atmosphere–5% CO₂ in 10 ml RPMI 1640 containing 11.1 mM glucose supplemented with 10% FBS, 2.06 mM glutamine, 100 IU/ml penicillin G, and 100 μ g/ml streptomycin (all Gibco-BRL).

Insulin secretion from islets

After overnight incubation, mouse islets were incubated in RPMI 1640 with 11.1 mM glucose in the presence of 1, 5, and 10 mM leucine, respectively, the normal RPMI 1640 containing 0.38 mM leucine as control (all the following experiments take 0.38 mM leucine as control). After 72-h leucine treatment, islets were rinsed once with M-KRB supplemented with 3.3 mM glucose and 0.1% human serum albumin and preincubated for 30 min in M-KRB at 37 °C. Consequently, a single islet was handpicked and incubated in 100 μ l M-KRB with 3.3 or 16.7 mM glucose. After 60 min of incubation in normal atmosphere at 37 °C, the medium (50 μ l) was collected and frozen for analysis of insulin.

Cell culture

INS-1E cells (generous gift from Prof. Claes B Wollheim, Geneva, Switzerland) were cultured in RPMI 1640 medium containing 11.1 mM D-glucose and supplemented with 10% FBS, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μ M 2-mercaptoethanol, at 37 °C in a humidified atmosphere containing 95% air and 5% CO₂. The cells were passaged weekly.

Insulin secretion from INS-1E cells

The INS-1E cells were seeded (3.0×10^5 cells/well) onto 24-well Black Visiplate TC (Wallac Oy, Turku, Finland) plates in 1 ml RPMI 1640. After adhering overnight, the cells were cultured in RPMI 1640 with 11.1 mM glucose in the presence of 0.38, 1, 5, and 10 mM leucine for 72 h in a humidified atmosphere (5% CO₂ 95% air at 37 °C). Afterward, the cells were preincubated with M-KRB supplemented with 3.3 mM glucose and 0.1% human serum albumin for 30 min and then the cells were incubated in 1 ml M-KRB containing 3.3 or 16.7 mM glucose for 1 h. Subsequently, supernatants (300 μ l) were collected, centrifuged, and 200 μ l were kept at –20 °C for insulin analysis. After the secretion study, the cells were washed once with 1 ml PBS (Gibco), and the number of cells was estimated using nuclear staining with SYTO 24 reagent (20 μ l/well) and measured by FLUOstar Galaxy (BMG, Ramcon, Denmark). Unless otherwise stated, passage numbers between 60 and 82 were used.

Insulin output of INS-1E cells

INS-1E cells were incubated and treated as earlier. For the insulin output study, incubation medium (25 μ l) was sampled after 24, 48, and 72 h and stored at -20°C until insulin analysis.

Insulin content of INS-1E cells

The INS-1E cells were incubated and treated as earlier, but in six-well plates with a density of 1.0×10^6 cells/well in 3 ml RPMI 1640. After 72 h, the cells were washed once with 2 ml cold PBS and then 1 ml glycine-BSA buffer was added. Cells were scratched and sonicated twice at 0°C for 14 s (Branson Sonifier 250, Danbury, CT, USA). We took 250 μ l to evaluate the total protein with a detergent-compatible protein kit (Bio-Rad Laboratories) for calibration of insulin content. The remaining 750 μ l was centrifuged for 30 min at 21460 g and the supernatant was collected and frozen at -20°C for a later insulin assay.

Insulin assay

Insulin was analyzed by RIA using a guinea pig antiporcine insulin antibody (Novo Nordisk, Bagsvaerd, Denmark) and mono- ^{125}I -(Tyr A14)-labeled human insulin (Novo Nordisk A/S) as tracer and rat insulin as standard (Novo Nordisk A/S). The separation of bound and free radioactivity was performed using ethanol. The intra- and interassay coefficients of variation were determined to be $<10\%$.

Determination of triglyceride and CH content

INS-1E cells were plated at 3.0×10^5 cells/well in 1 ml of medium in 24-well Black Visiplate TC plates. The cells were allowed to adhere overnight and then incubated and treated as earlier. After 72 h, the cells were washed once with 1 ml PBS, and the number of cells was estimated as earlier. The medium was removed, and the cells were frozen for 1 h at -80°C . Subsequently, the cells were treated for 20 min with triglyceride (TG) reagents (250 μ l/well, Roche) at room temperature. TG content was determined with a TG GPO-PAP kit (Roche) and normalized to cell number. The recovery of TG was $\sim 90\%$.

Correspondingly, cells were treated for 20 min with CH reagents (250 μ l/well, Roche) at room temperature. CH content was determined by a CH CHOD-PAP kit (Roche) and was normalized to cell number. The recovery of CH content was $\sim 90\%$.

^3H -thymidine incorporation in INS-1E cells

^3H -thymidine incorporation was used to monitor INS-1E cell proliferation and DNA synthesis. Briefly, INS-1E cells (4×10^4 /well) were seeded in 96-well isoplates (Wallac Oy) and cultured in RPMI 1640 with 11.1 mM glucose and

10% FBS. On the next day, the cells were incubated with 'starvation medium' (RPMI 1640 with 0.5 mM glucose, 0.1% BSA, without FBS) for 24 h. The cells were then cultured for another 72 h in RPMI 1640 containing 0.5% BSA, 11.1 mM glucose in the presence of 0.38, 1, 5, and 10 mM leucine, and 1 μCi [methyl- ^3H]thymidine (Perkin Elmer, Wellesley, MA, USA). After 72 h, the cells were washed twice in cold PBS and 200 μ l liquid scintillator was applied. The incorporated [methyl- ^3H]thymidine was counted by a 1450 MicroBeta TRILUX (Wallac).

The incidence of dead INS-1E cells

INS-1E cells were seeded in 96-well Black Visiplate TC plates (Wallac Oy) at a density of 4×10^4 cells/well in 200 μ l medium, the cells were allowed to adhere overnight, and then treated and cultured with different concentrations of leucine as earlier. After 72 h, the number of dead cells in each well was calculated using a fluorometric assay kit based on the cell lysis and staining method (Sakai *et al.* 2001; Cytotoxic Fluoro-test Wako; Wako Pure Chemical Industries, Osaka, Japan) in the FLUOstar Galaxy. A linear relationship was confirmed in advance between the viable cell density and fluorescence intensity.

RNA extraction and cDNA synthesis

Three different passages of INS-1E cells were incubated in RPMI 1640 in the presence of 0.38 or 5 mM leucine for 72 h. The RNA of treated cells was extracted using the AllPrep RNA/Protein kit (Qiagen) according to the manufacturer's instructions (AllPrep RNA/Protein Handbook; Qiagen) on the QIAcube machine (Qiagen). RNA concentration and purity were determined by measuring the absorbance at 260 and 280 nm (NanoDrop ND-8000 u.v.-Vis Spectrophotometer, NanoDrop Technologies Wilmington, DE, USA). The RNA quality was evaluated by examining the 18s and 28s ribosomal band on a 1% nondenaturing agarose gel, stained with SYBR green.

RT was performed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions. We applied 1 μg total RNA for each 20 μ l RT reaction.

Quantitative RT-PCR

RT-PCR was performed using an Applied Biosystems 7500 FAST PCR machine (Applied Biosystems). Predesigned TaqMan probes and primers with the following catalog numbers were obtained from Applied Biosystems: *Pdx1* (Rn00755591_m1), the v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (*MafA*, Rn00845206_s1), insulin 1 (*Ins1*, Rn02121433_g1), insulin 2 (*Ins2*, Rn01774648_g1), acetyl-CoA carboxylase α (*Acc1* (*Acaca*), Rn00573474_m1), fatty acid synthase (*Fasn*, Rn01463548_m1), sterol-regulatory element binding factor 2 (*Srebf2*, Rn01306296_m1),

3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*, Rn00565598_m1), mevalonate (diphospho) decarboxylase (*Mvd*, Rn00579216_m1), squalene epoxidase (*Sqle*, Rn00567532_m1), LDL receptor (*Ldlr*, Rn00598442_m1), cytochrome *c* oxidase (*Cox*), subunit VIa, polypeptide 2 (*Cox6a2*, Rn00563091_g1), caspase-3 (*Casp3*, Rn00563902_m1), death-associated protein kinase 3 (*Dapk3*, Rn00574826_m1), Bcl2 modifying factor (*Bmf*, Rn00594968_m1), and eukaryotic 18S (*Hs9999901_s1*).

The RT-PCR mix contained 5 μ l of 2 \times TaqMan FAST Universal Master Mix (P/N 43660783, Applied Biosystems), 0.5 μ l of 20 \times TaqMan Assay/probe (Applied Biosystems), and cDNA equivalent to 50 ng total RNA in 4.5 μ l H₂O. The thermal FAST cycle program was one cycle at 95 °C for 20 s, 40 cycles at 95 °C for 3 s, and 60 °C for 30 s. Each PCR sample was performed in six repeats, and gene expressions were normalized to eukaryotic 18S expression. All assays were carried out in 96-well plates with an optical adhesive cover (P/N 4346906 and P/N 4311971, Applied Biosystems). We used the $2^{-\Delta\Delta CT}$ method to calculate the relative gene expression (Pfaffl 2001). No template controls and no amplification controls were included for each gene as negative controls.

INS-1E cell growth and lysis for proteomics

Protein from three different passages of INS-1E cells was extracted at the same time as RNA was purified on the QIAcube machine. We took two samples every time for each condition; as a result, there were six replicates of each treatment used for proteomics. The protein content of the cell suspensions was analyzed by the Bradford assay (Bio-Rad). In order to obtain sufficient protein concentration of the cell suspensions, the samples were precipitated with addition of 250 μ l 24% trichloroacetic acid at 4 °C and left to precipitate for 30 min at 0 °C, followed by centrifugation at 6000 *g* at 0 °C for 10 min. The supernatant was removed, the protein pellet was washed twice by addition of 500 μ l acetone at 0 °C, and centrifuged as described between each washing step. The protein pellets were kept at -80 °C for proteomic analysis.

Proteomic analyses The 2-dimensional gel electrophoresis (2-DGE) analysis was performed essentially as described earlier (Young *et al.* 2010). Briefly, the stored protein pellets were thawed and solubilized in 250 μ l of 2D lysis buffer and subsequently tenfold in Millipore water. Based on protein determination using the Bradford assay, a sample volume corresponding to 100 μ g protein was applied to each gel. The 12 lysed cell samples were analyzed in a single 2-DGE gel set consisting of six gels representing either control INS-1E cells or treated with leucine, respectively, as described earlier using 11 cm IPG strips (pH 5–8) for the first dimension and 12.5% Criterion gels (Bio-Rad) for the second dimension. Analytical gels were stained with Flamingo Pink (Bio-Rad) and scanned using a Molecular Imager FX (Bio-Rad). Images were analyzed with PDQuest (Bio-Rad). Gels for spot

identification were stained with colloid Coomassie Blue R-250 (Kang *et al.* 2002). In-gel digestion for peptide mass fingerprinting, desalting, and concentration of protein spots, identification of MFGM proteins by matrix-assisted laser desorption ionization–time of flight mass spectrometry, and image analysis was performed as described earlier (Larsen *et al.* 2010).

Statistical analysis

Data are presented as mean \pm s.e.m. We compared groups using one-way ANOVA; we also performed statistical analysis using the unpaired two-tailed Student's *t*-test with unequal

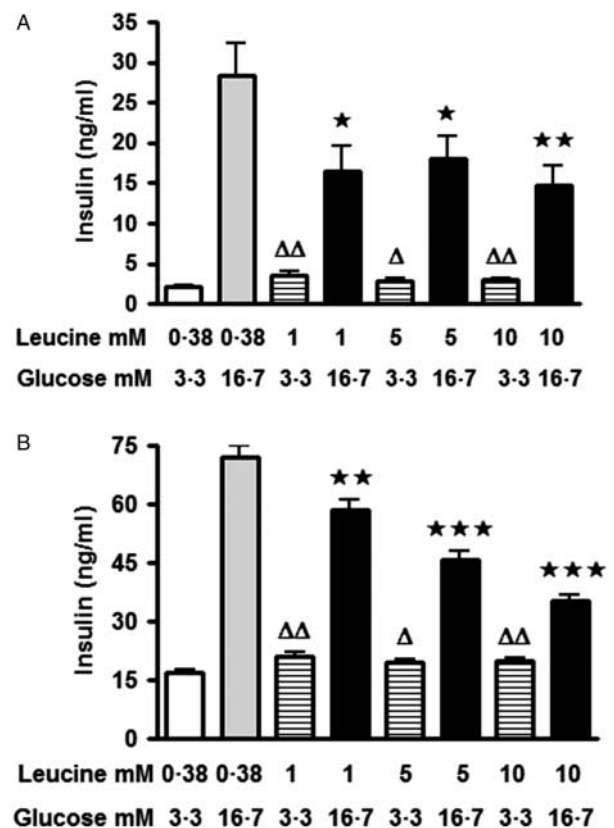


Figure 1 (A) Effects of 72-h incubation with leucine on the response of mouse islets to glucose. The insulin secretion from mouse islets at low (3.3 mM) ($\Delta P < 0.05$, $\Delta\Delta P < 0.01$) and high (16.7 mM) glucose concentrations ($*P < 0.05$ and $**P < 0.01$) for 1 h compared with control (0.38 mM leucine). This part includes three independent experiments. For each experiment, we applied the mean value from 8 to 11 islets for each condition. (B) Effects of 72-h incubation with leucine on the response of INS-1E cells to glucose. The insulin secretion from per 10 000 INS-1E cells at low (3.3 mM) ($\Delta P < 0.05$, $\Delta\Delta P < 0.01$) and high (16.7 mM) glucose concentrations ($**P < 0.01$, $***P < 0.001$) for 1 h compared with control (0.38 mM leucine). This part includes three independent experiments. For each experiment, we applied the mean value from six samples for each condition.

variances. Each treatment condition was compared with controls with Bonferroni's correction. Differences of $P < 0.05$ were considered to be significant. All statistical analysis was performed with GraphPad Prism Software (San Diego, CA, USA).

Results

Impact of leucine on insulin secretion from isolated islets and INS-1E cells

Figure 1A shows that at 3.3 mM glucose BIS from isolated islets increased after 72-h incubation with 1, 5, or 10 mM leucine. By contrast, glucose (16.7 mM)-stimulated insulin secretion decreased after 72-h exposure of the islets to 1, 5, or 10 mM leucine (Fig. 1A). Similar results were obtained in INS-1E cells, demonstrating increased BIS and decreased GSIS after 72-h exposure to high leucine concentrations (Fig. 1B). We did not find any change in insulin content after 72-h exposure to 1, 5, and 10 mM leucine compared with control (0.928 ± 0.129 ng/ μ g protein (control), 0.812 ± 0.136 ng/ μ g protein (1 mM leucine), 0.832 ± 0.107 ng/ μ g protein (5 mM), 0.884 ± 0.131 ng/ μ g protein (10 mM)); after 72 h, no change in insulin output was

detected with 1, 5, and 10 mM leucine (386.7 ± 10.5 ng/ml per 10 000 cells (control), 388.8 ± 16.4 ng/ml per 10 000 cells (1 mM leucine), 365.2 ± 7.2 ng/ml per 10 000 cells (5 mM), and 392.4 ± 11.0 ng/ml per 10 000 cells (10 mM)).

Effects of 72-h exposure to leucine on gene expressions in INS-1E cells

The impact of 72-h incubation with 5 mM leucine on gene expression was studied in INS-1E cells for a number of β -cell genes (Fig. 2A, B, C, D, E, F, G, H, I, J, K, and L). Leucine upregulated *Pdx1* expression (Fig. 2A), did not change *MafA* expression (Fig. 2B), downregulated *Ins1* expression (Fig. 2C), and upregulated *Ins2* expression (Fig. 2D). As shown in Fig. 2E and F, leucine increased *Acaca* and *Fasn* expressions in INS-1E cells.

As depicted in Fig. 2G, leucine significantly increased *Srebf2* mRNA expression. Figure 2H, I and J demonstrate changes in the expression of a series of genes encoding for enzymes involved in CH biosynthesis in INS-1E cells exposed to leucine. *Hmgcr*, *Mvd*, and *Sqle* were upregulated. Leucine also increased *Ldlr* mRNA level in INS-1E cells (Fig. 2K). By contrast, leucine pronouncedly reduced *Cox6a2* mRNA level in INS-1E cells (Fig. 2L).

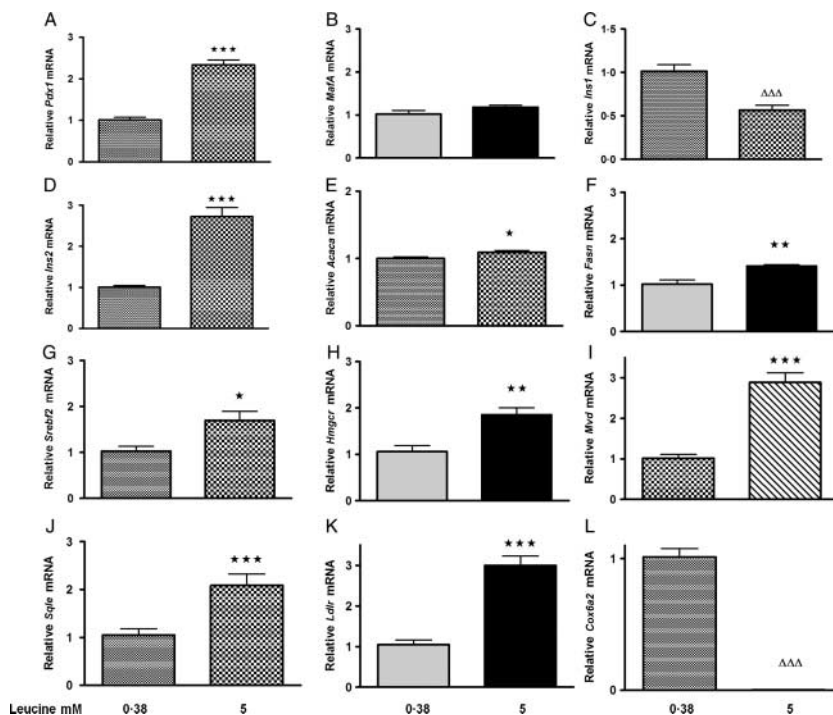


Figure 2 Changes in gene expressions in INS-1E cells induced by 5 mM leucine. The following genes were investigated: *Pdx1* (A), *MafA* (B), *Ins1* (C), *Ins2* (D), *Acaca* (E), *Fasn* (F), *Srebf2* (G), *Hmgcr* (H), *Mvd* (I), *Sqle* (J), *Ldlr* (K), and *Cox6a2* (L). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control, $\Delta\Delta\Delta P < 0.001$ vs control. RNA extraction includes three experiments applying different passage number cells. RT-PCR was performed in six repeats.

Influence of leucine on TG and CH content in INS-1E cells

As seen in Fig. 3A, 72-h incubation with leucine (1, 5, or 10 mM) reduced the TG content of INS-1E cells significantly. Figure 3B demonstrates that the CH content in INS-1E cells was increased after 72-h culture with 1, 5, or 10 mM leucine respectively.

Effect of leucine on gene expressions associated with apoptosis in INS-1E cells

Leucine (5 mM) did not affect the mRNA levels of *Casp3* (Fig. 4A) and *Dapk3* (Fig. 4B) in INS-1E cells, whereas the pro-apoptotic protein *Bmf* (Fig. 4C) mRNA was downregulated.

Impact of leucine on the viability of INS-1E cells and 3 H-thymidine incorporation in INS-1E cells culture

While the percentage of dead INS-1E cells was unchanged by 1 and 5 mM leucine, 10 mM leucine augmented the number of dead INS-1E cells (Fig. 5A). Leucine (1, 5, and 10 mM)

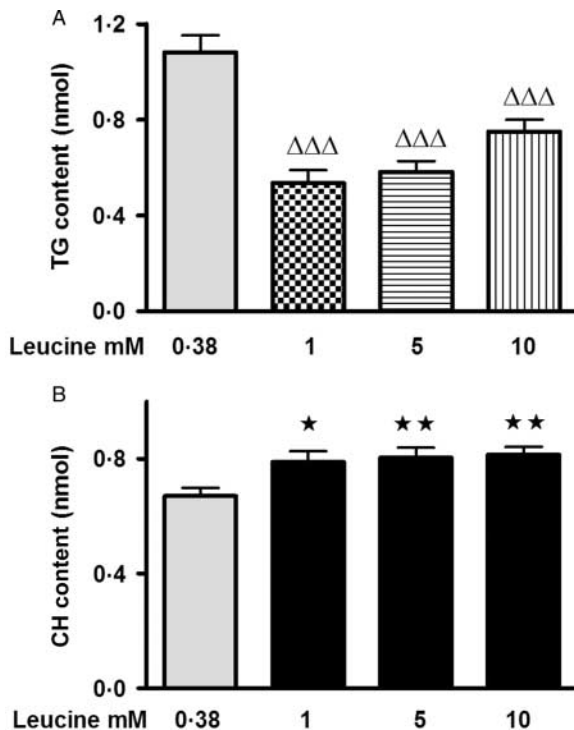


Figure 3 (A) Effect of leucine on TG content of INS-1E cells. Changes in TG content per 10 000 INS-1E cells after 72-h incubation with leucine (1–10 mM). $\Delta\Delta\Delta P < 0.001$ vs control. This part includes three independent experiments. For each experiment, we applied the mean value from six samples for each condition. (B) Effect of leucine on CH content of INS-1E cells. Changes in CH content per 10 000 INS-1E cells after 72-h incubation with 1–10 mM leucine, $*P < 0.05$, $**P < 0.01$ vs control. This part includes three independent experiments. For each experiment, we applied the mean value from six samples for each condition.

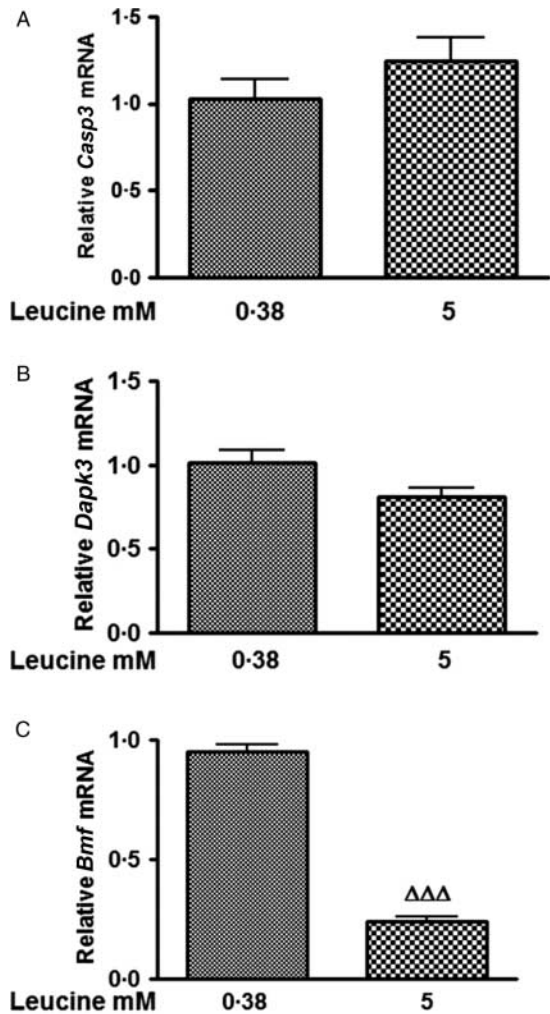


Figure 4 Impact of leucine on the expressions of genes associated with apoptosis. Alterations of mRNA expression in genes associated with apoptosis in INS-1E cells induced by 72 h exposure to 5 mM leucine. *Casp3* (A), *Dapk3* (B), *Bmf* (C), $\Delta\Delta\Delta P < 0.001$ vs control. RNA extraction includes three independent experiments. RT-PCR was performed in six repeats.

decreased 3 H-thymidine incorporation in INS-1E cells, indicating a leucine-induced suppression of INS-1E cell proliferation (Fig. 5B).

Proteomics studies: alteration in proteins in INS-1E cells induced by leucine

Results from proteomic studies are presented in Table 1. Leucine (5 mM) increased the autophagy-related enzyme, cathepsin D. Leucine decreased serine/threonine-protein phosphatase PP1, which is related to phosphorylation/dephosphorylation states of proteins. Leucine decreased GTP-binding nuclear protein and increased dihydropyrimidinase-related protein 2 (DRP2), which are related to ISG

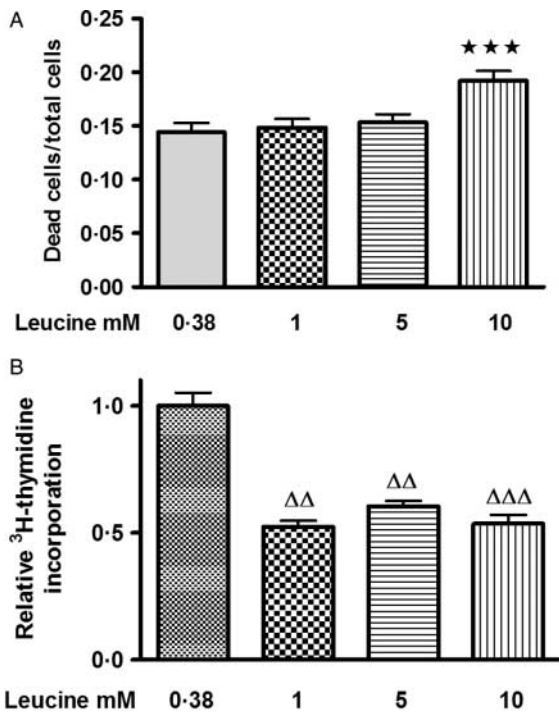


Figure 5 (A) Impact of leucine on the viability of INS-1E cells. Impact of 72-h incubation with leucine (1–10 mM) on the viability of INS-1E cells, *** $P < 0.001$ vs control. This part includes three independent experiments. For each experiment, we applied the mean value from ten samples for each condition. (B) Effect of leucine on the incorporation of ³H-thymidine into INS-1E cells. Effect of 72-h incubation with leucine (1–10 mM) on ³H-thymidine incorporation in INS-1E cells, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ vs control. This part includes three independent experiments. For each experiment, we applied the mean value from ten samples for each condition.

transport/trafficking. Proteasome subunit α type 6 was identified in two spots, one was upregulated, while the other was downregulated; the two spots are probably active and inactive forms of proteasome subunit α type 6.

Discussion

In this study, we demonstrated that long-term exposure to leucine caused β -cell dysfunction with increased BIS and decreased GSIS in both mouse islets and INS-1E cells respectively. The functional derangement of β cells took place concomitantly with alterations in a large number of β -cell gene expressions. For example, *Ins1* gene expression was downregulated by chronic exposure to high leucine levels (Fig. 2C), being in line with the observation that GSIS was inhibited and that *Ins1* accounts for 60–90% of total insulin immunoreactivity in β -cell lines (Linde *et al.* 1993). The expressions of the two insulin genes have been proposed to be independently regulated as well as coordinately regulated in both rats and mice (Linde *et al.* 1993). The differential effect of

leucine on expressions of *Ins1* and *Ins2* mRNA (Fig. 2D) appears puzzling. There was no change in total insulin content or output in INS-1E cells. *Pdx1* and *MafA* are potent stimulators of the transcription of insulin genes (Docherty *et al.* 2005), and the increased *Pdx1* mRNA expression found in this study (Fig. 2A) is very interesting; it is in accordance with increased mRNA expression of *Pdx1* in isolated islets from T2D patients, despite less insulin release than control islets in response to glucose (Del Guerra *et al.* 2005); furthermore, it has previously been shown that glutamine upregulates *Pdx1* expression in BRIN-BD11 cells (Corless *et al.* 2006).

In a global gene expression profiling study (Liu *et al.* 2011), the largest cluster of genes in INS-1E cells induced by exposure to excess leucine is those regulating CH metabolism. AMP-activated protein kinase (AMPK) was downregulated (Liu *et al.* 2011), *Sreb2* mRNA expression was enhanced (Figs 2G and 6), and as many as ten enzymes in the CH biosynthetic pathway were upregulated (Liu *et al.* 2011). In line with the change of AMPK in INS-1E cells after prolonged exposure to leucine, islets from T2D patients demonstrated that the percentage of activated AMPK protein was significantly lower than control islets (Del Guerra *et al.* 2005). The principal targets for phosphorylation by AMPK are *Hmgcr* and *Acaca* (Towler & Hardie 2007). Interestingly, *Sreb2* gene is tightly associated with the regulation of a number of genes encoding for enzymes involved in the CH biosynthetic pathway (Ishikawa *et al.* 2008). Our RT-PCR results showed that leucine upregulated expressions of CH biosynthetic enzymes, e.g. acetyl-CoA acetyltransferase 2, 3-hydroxy-3-methylglutaryl-CoA synthase 1 (Liu *et al.* 2011), *Hmgcr*, *Mvd*, *Sqle* (Fig. 2H, I, and J), and *Ldlr* (Fig. 2K). In line with this, we found that the CH content was significantly increased in INS-1E cells chronically exposed to elevated leucine levels (Fig. 3B). The series of events

Table 1 INS-1E cellular proteins identified by MS and significantly affected by leucine. Fold change (FC) in mean relative spot volumes was calculated relative to the control, FC > 1 indicates upregulation, FC < 1 indicates downregulation

Protein name	Swiss Prot Acc. no. ^a	Mouse score ^b	t-test (P)	Fold change
Pyruvate dehydrogenase E1 component subunit β	P49432	73*	0.02	1.3
Cathepsin D	P24268	59	0.002	1.5
Serine/threonine-protein phosphatase PP1	P62138	115*	0.04	0.9
Dihydro pyridinase-related protein 2	P47942	70*	0.03	1.4
Proteasome subunit α type 6	P60901	89*	0.03	0.8
Proteasome subunit α type 6	P60901	82*	0.0003	2.1
GTP-binding nuclear protein	P62828	65*	0.05	0.5

^aPrimary accession key in the SWISS-PROT database.

^bScore of the Mascot search. Significant scores ($P < 0.05$) are marked by *.

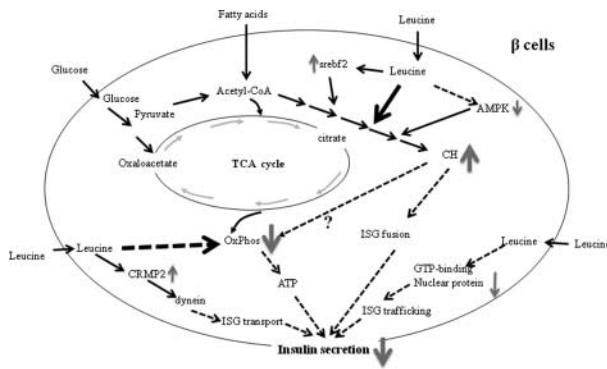


Figure 6 Scheme depicting major pathways relating to the possible effects of leucine on pancreatic β cells. AMPK, AMP-activated protein kinase; CH, cholesterol; CRMP-2, collapsin response mediator protein 2; ISGs, insulin secretory granules; OxPhos, oxidative phosphorylation; SREBF2, sterol-regulatory element binding factor 2; TCA, tricarboxylic acid. Dashed line arrow indicates negative effect, solid line arrow indicates positive effect.

described here is coherent with several data obtained *in vitro* and *in vivo* in animal models and have recently been put into perspective (Peyot *et al.* 2010, Vergeer *et al.* 2010, Kruit *et al.* 2012). Combined deletion of ATP-binding cassette transporter G subfamily 1 (ABCG1) and the ATP-binding cassette transporter subfamily A member 1 (ABCA1) resulted in increased islet CH levels compared with control, β -cell-specific ABCA1 deficiency, and globally deficient ABCG1; notably, islets lacking both ABCA1 and ABCG1 had an even greater reduction in GSIS compared with islets from mice lacking either ABCA1 or ABCG1 (Kruit *et al.* 2012). Likewise, high-fat induced obese mice were divided into low responders (LDR) and high responders (HDR) according to their body weight (Peyot *et al.* 2010). There was a 90% and 60% increase in free CH in islets from HDR and LDR respectively; LDR mice islets displayed impaired GSIS, whereas GSIS of HDR mice islets was almost blunted. Moreover, in ABCA1 heterozygotes, the first-phase insulin response is significantly impaired (Vergeer *et al.* 2010).

Consistent with the downregulation of AMPK, long-term exposure to extra leucine results in upregulation of *Acaca* and *Fasn* mRNA expressions in INS-1E cells (Fig. 2E and F). Corless *et al.* (2006) previously also found increased *Acaca* expression to glutamine. These suggest that leucine may inhibit FA β oxidation and favor CH and FA synthesis. To our surprise, increased levels of leucine resulted in TG reduction in INS-1E cells (Fig. 3A). One explanation might be that leucine strongly induced an increase in CH biosynthesis and is associated with higher consumption of substrate. This may result in a shortage of substrate for TG synthesis and a reduction in TG content.

INS-1E cells demonstrated a robust suppression of *Cox6a2* gene expression assessed by both microarray (Liu *et al.* 2011) and RT-PCR (Fig. 2L) after prolonged exposure to excess leucine (Fig. 6). Cox represents the terminal and rate-limiting enzyme complex of the OxPhos pathway, which is

responsible for ATP production in mitochondria. The critical regulatory role of ATP production by OxPhos is underscored by the observation that the Cox activity of islets from the transgenic MKR mouse (a dominant-negative IGF1 receptor mutation specifically in skeletal muscle) was significantly reduced (Lu *et al.* 2010). As a result, the ATP/ADP ratio was blunted in response to high glucose, and this was followed by a blocking of GSIS, indicating a reduced mitochondrial oxidative capacity (Lu *et al.* 2010). Accordingly, advanced glycation end products cause impaired GSIS from mice islets and INS-1 cells by inhibiting Cox activity and ATP synthesis (Zhao *et al.* 2009). This is in line with observations in human diabetes. The expression of a co-regulated subset of OxPhos in individuals with IGT was also downregulated, indicating that downregulation of a co-regulated subset of OxPhos precedes onset of T2D (Mootha *et al.* 2003). Taken together, this highlights Cox as an important mechanism by which leucine impairs GSIS in INS-1E cells. Previously, clonal BRIN-BD11 β cells exposed to alanine for 24 h exhibited impaired alanine-induced insulin secretion (Cunningham *et al.* 2005). Intriguingly, in CH-enriched β cells, the glucose-mediated increase in cellular ATP content was dramatically reduced, and this was related to a decrease in glucose uptake via glucose transporter 2 and an impairment of mitochondrial metabolism (Lee *et al.* 2011). The relationship between leucine-induced CH accumulation and leucine-induced Cox downregulation should be further investigated (Fig. 6). Cox activity is also regulated by phosphorylation and dephosphorylation. As many as at least ten of the 13 subunits of Cox could be phosphorylated at serine and/or threonine residues (Thompson 2002). As seen from our proteomics results, we found that serine/threonine-protein phosphatase 1 decreased in leucine-treated INS-1E cells (Table 1). Its significance should be further explored deeply.

DRP2 also known as collapsin response mediator protein 2 (CRMP2) plays a role in the maintenance of intracellular Ca^{2+} homeostasis by direct binding to cytoplasmic loops of N-type Ca^{2+} channels (Wang *et al.* 2010). Moreover, CRMP2 directly bound cytoplasmic dynein and prevented dynein-driven microtubule transport in COS-7 cells (Arimura *et al.* 2009). Leucine significantly increased CRMP2 content in INS-1E cells (Table 1), indicating a potentially detrimental effect on insulin secretion (Fig. 6), which needs to be corroborated in β cells. Furthermore, by the application of proteomics, we found that leucine reduced the expression of GTP-binding nuclear protein Ran (Table 1) in INS-1E cells, which has been reported to be involved in the regulation of microtubule assembly (Dikovskaya *et al.* 2010). In accordance with our findings, elevated islet CH accumulation due to a lack of β -cell ABCA1 directly impairs depolarization-induced exocytotic fusion of ISGs (Kruit *et al.* 2011; Fig. 6). Leucine-induced cellular CH accumulation, CRMP2 upregulation, and GTP-binding nuclear protein downregulation are new findings and deserve further evaluation. These findings may have relevance for ISG exocytotic fusion, ISG transport, and trafficking in pancreatic β cells (Fig. 6).

Another protein identified as being differentially regulated by leucine was proteasome subunit α type 6 (Table 1). Interestingly, insulin receptor substrate 1 (IRS1) is selectively regulated by a proteasome degradation pathway activated during prolonged insulin exposure. This is accomplished through regulation of the amount of IRS1, an important receptor-related component in the insulin-signaling cascade (Zhande *et al.* 2002). Furthermore, leucine diminished insulin receptor expression (Liu *et al.* 2011) and upregulated ribosomal protein S6 kinase polypeptide 1 mRNA level in INS-1E cells (Liu *et al.* 2011), the latter inhibited serine phosphorylation of IRS1 to impair insulin action (Tremblay *et al.* 2005). Consequently, leucine appears to induce IR through multiple mechanisms (Tremblay *et al.* 2005, Balage *et al.* 2011).

Currently, there is doubt that apoptosis is a major β -cell death mechanism in T2D. We found no indication that leucine affects INS-1E cell death at the concentrations of 1 and 5 mM (Fig. 5A), results that are in agreement with Zhang *et al.* (2009). This was further supported by the gene expressions of key mediators in apoptosis using RT-PCR. Thus, 5 mM leucine did not change *Casp3* (Fig. 4A) and *Dapk3* (Fig. 4B) expressions in INS-1E cells. Moreover, leucine significantly downregulated *Bmf* expression in INS-1E cells (Fig. 4C), suggesting an anti-apoptosis effect of leucine. The amount of β -cell mass, at any given moment, is represented by the sum of replication, size, and neogenesis minus the rate of apoptosis. The ^3H -thymidine incorporation experiment showed that INS-1E cell proliferation was obviously reduced by exposure to elevated leucine levels for 72 h (Fig. 5B). This suggests that elevated leucine may cause reduced β -cell mass by decreasing β -cell mitotic division and proliferation.

In conclusion, chronic exposure to elevated levels of leucine induces β -cell dysfunction, with increased BIS and decreased GSIS in both isolated mouse islets and clonal β cells. Accordingly, long-term exposure of INS-1E cells to excess leucine resulted in abnormal CH metabolism, impaired OxPhos, and insulin signal transduction. Overall, these observations indicate that exposure to leucine may have detrimental effects on both β -cell function and insulin sensitivity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Z L, P B J, S G, and K H designed this project, Z L completed experiments described in this manuscript. L B L completed proteomics experiment. P B J and S G provided experimental advice. Z L wrote the manuscript; K H, P B J, S G, and L B L helped with manuscript revision.

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