Diabetologia (2010) 53:1395–1405
DOI 10.1007/s00125-010-1707-y

ARTICLE

Palmitate induces a pro-inflammatory response in human pancreatic islets that mimics *CCL2* expression by beta cells in type 2 diabetes

M. Igoillo-Esteve · L. Marselli · D. A. Cunha · L. Ladrière · F. Ortis · F. A. Grieco · F. Dotta · G. C. Weir · P. Marchetti · D. L. Eizirik · M. Cnop

Received: 28 November 2009 / Accepted: 27 January 2010 / Published online: 6 April 2010

Abstract
Aims/hypothesis Beta cell failure is a crucial component in the pathogenesis of type 2 diabetes. One of the proposed mechanisms of beta cell failure is local inflammation, but the presence of pancreatic islet inflammation in type 2 diabetes and the mechanisms involved remain under debate.

Methods Chemokine and cytokine expression was studied by microarray analysis of laser-capture microdissected islets from pancreases obtained from ten non-diabetic and ten type 2 diabetic donors, and by real-time PCR of human islets exposed to oleate or palmitate at 6 or 28 mmol/l glucose. The cellular source of the chemokines was analysed by immunofluorescence of pancreatic sections from individuals without diabetes and with type 2 diabetes.

Results Microarray analysis of laser-capture microdissected beta cells showed increased chemokine and cytokine expression in type 2 diabetes compared with non-diabetic controls. The inflammatory response in type 2 diabetes was mimicked by exposure of non-diabetic human islets to palmitate, but not to oleate or high glucose, leading to the induction of IL-1β, TNF-α, IL-6, IL-8, chemokine (C-X-C motif) ligand 1 (CXCL1) and chemokine (C-C motif) ligand 2 (CCL2). Interference with IL-1β signalling abolished palmitate-induced cytokine and chemokine expression but failed to prevent lipotoxic human islet cell death. Palmitate activated nuclear factor κB (NF-κB) in human pancreatic beta and non-beta cells, and chemically induced endoplasmic reticulum stress caused cytokine expression and NF-κB activation similar to that occurring with palmitate.

Conclusions/interpretation Saturated-fatty-acid-induced NF-κB activation and endoplasmic reticulum stress may contribute to IL-1β production and mild islet inflammation in type 2 diabetes. This inflammatory process does not contribute to lipotoxicity ex vivo, but may lead to local chemokine release.

Keywords Chemokine · Cytokine · Endoplasmic reticulum · Fatty acid · Inflammation · Interleukin-1β · Islet · Palmitate · Pancreatic beta cell · Type 2 diabetes

M. Igoillo-Esteve and L. Marselli contributed equally to this study.

Electronic supplementary material The online version of this article (doi:10.1007/s00125-010-1707-y) contains supplementary material, which is available to authorised users.

M. Igoillo-Esteve · D. A. Cunha · L. Ladrière · F. Ortis · D. L. Eizirik · M. Cnop (✉)
Laboratory of Experimental Medicine,
Université Libre de Bruxelles,
CP-618, Route de Lennik 808,
1070 Brussels, Belgium
e-mail: mcnop@ulb.ac.be

L. Marselli · P. Marchetti
Department of Endocrinology and Metabolism,
Metabolic Unit–University of Pisa,
Pisa, Italy

F. A. Grieco · F. Dotta
Department of Internal Medicine, Endocrine and Metabolic Sciences and Biochemistry, University of Siena
and Umberto Di Mario Foundation,
Siena, Italy

G. C. Weir
Joslin Diabetes Center, Harvard Medical School,
Boston, MA, USA

M. Cnop
Division of Endocrinology, Erasmus Hospital,
Université Libre de Bruxelles,
Brussels, Belgium

Abbreviations

ATF6	Activating transcription factor 6
CCL	Chemokine (C-C motif) ligand
CPA	Cyclopiazonic acid
CXCL1	Chemokine (C-X-C motif) ligand 1
eIF2α	Eukaryotic translation initiation factor 2, subunit 1 α
ER	Endoplasmic reticulum
IκBα	Nuclear factor κB inhibitor, α
IRE1	Inositol-requiring 1
IL-1ra	IL-1 receptor antagonist
LCM	Laser capture microdissection
ND	Non-diabetic
NF-κB	Nuclear factor κB
PERK	PRKR-like endoplasmic reticulum kinase
TLRs	Toll-like receptors

Introduction

Insulin deficiency and insulin resistance contribute to the development of type 2 diabetes. Pancreatic beta cell dysfunction is present early in the pathogenesis of type 2 diabetes and worsens over time [1], at least in part as a result of loss of functional beta cell mass [2]. By increasing insulin requirements, insulin resistance can precipitate the onset of hyperglycaemia. Both beta cell failure and insulin resistance are determined by genetic and environmental factors, the latter causing the rapidly increasing prevalence of type 2 diabetes worldwide. Sedentary lifestyle and energy-dense diets rich in refined sugars and saturated fats are known risk factors for type 2 diabetes, but the mechanisms involved are only partially understood.

Glucotoxicity and lipotoxicity are terms coined for the deleterious effects of high circulating concentrations of glucose and lipids, both at the level of insulin sensitivity and in beta cells. Chronically elevated glucose concentrations affect beta cell function and survival through increased generation of reactive oxygen species and mitochondrial dysfunction, endoplasmic reticulum (ER) stress, and JNK signalling [3–5]. NEFA, and in particular saturated NEFA, impair beta cell function and cause apoptosis through ceramide synthesis, JNK activation and oxidative and ER stress [5–9]. Saturated NEFA cause ER stress by depleting ER calcium, which secondarily affects protein folding in the organelle [10]. Human islets exposed in vitro to palmitate activate the three branches of the ER stress response under the control of PRKR-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring 1 (IRE1) [10]. Markers of oxidative [11] and ER stress [8, 12, 13] are present in islets of type 2 diabetic patients, suggesting these stress responses may indeed play a role in the development and progression of the disease.

Mild inflammation by activation of the innate immune system has been suggested to play a role in the pathogenesis of type 2 diabetes, and it is present years before disease onset [14]. In the adipose tissue of obese individuals, infiltrating macrophages produce cytokines, modulate adipocyte adipokine secretion and contribute to the development of insulin resistance [15]. The spill over of inflammatory cytokines into the circulation has been associated with the development of type 2 diabetes. While the single increase in circulating IL-6 does not enhance disease risk, the combined elevation of IL-6 and IL-1β is associated with a threefold increased risk for type 2 diabetes [16]. A new role for inflammation in type 2 diabetes has been recently proposed, namely as a trigger of beta cell dysfunction and death [17]. Macrophage infiltration was observed in islets from type 2 diabetic individuals compared with non-diabetic (ND) individuals, with 20% of the type 2 diabetic islets containing >3 $CD68^+$ cells per islet compared with 5% of the ND islets [18, 19]; the role of these macrophages is unknown. It has been proposed that beta cells contribute to the inflammatory process in type 2 diabetes by their own secretion of IL-1β in response to high glucose. In a series of studies, it was suggested that glucotoxicity in human beta cells is mediated by Fas (TNF receptor superfamily, member 6) receptor upregulation and nuclear factor κB (NF-κB) activation, culminating in beta cell dysfunction and apoptosis [20, 21], though this was not confirmed by others [22, 23]. Altogether, the cause and role of islet inflammation in type 2 diabetes remains controversial and poorly understood.

We have used human pancreatic beta cells and islets from ND and type 2 diabetic individuals to answer the following questions: (1) is an inflammatory process present in type 2 diabetic beta cells, as evaluated by array analysis of laser-capture microdissected beta cells from type 2 diabetic and normoglycaemic individuals; (2) do NEFA and/or high glucose contribute to this process; (3) by which signalling events and cellular response mechanisms do saturated NEFA induce an inflammatory response in human islets; and (4) does NEFA-induced inflammation contribute to lipotoxicity? The results demonstrate the presence of mild inflammation in islets from type 2 diabetic individuals, a process that is mimicked by in vitro exposure of ND human islets to palmitate. The saturated-NEFA-induced inflammation could be a consequence of NEFA-induced ER stress and does not directly contribute to human islet lipotoxicity.

Methods

Laser-capture microdissection and microarray analysis Pancreatic samples from ten ND multiorgan donors (age

60±2 years, four women/six men, BMI 30.6±1.6 kg/m^2) and ten type 2 diabetic donors (age 67±7 years, three women/seven men, BMI 30.9±6.2 kg/m^2, known duration of diabetes 5.3±2.3 years, insulin/oral therapy 33%/66%) were obtained with approval of the Ethics Committee in Pisa, Italy. The causes of death were similar. The available clinical characteristics of the donors are provided in Electronic supplementary material (ESM) Table 1; no data were available on levels of circulating lipids or inflammatory markers. Laser-capture microdissection (LCM) was performed as previously detailed [24, 25]. Briefly, frozen pancreatic sections were fixed, dehydrated and air-dried. LCM was performed using PixCell II Laser Capture Microdissection System (Arcturus Engineering, Mountain View, CA, USA) by melting thermoplastic films mounted on transparent LCM caps (Arcturus) on beta cells, identified by their intrinsic autofluorescence, in islets with no signs of amyloid deposits. The microdissected beta cells were incubated in guanidine isothiocyanate extraction buffer and RNA was extracted by modification of the RNA micro-isolation protocol [24]. The total RNA underwent two amplification rounds using the RiboAmp HS RNA Amplification Kit (Arcturus) and was biotinylated using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY, USA). RNA products were fragmented and hybridised to GeneChip Human X3P Array (Affymetrix, Santa Clara, CA, USA). Array data were normalised and analysed using DNA-Chip Analyzer (dChip) software (available from http://biosun1.harvard.edu/complab/dchip/) that assesses the standard errors for the expression indices and calculates confidence intervals for fold changes. Lower confidence bound (used at a cut-off value of 1.2), a conservative estimate of the fold change, and the p value were used to assess differentially expressed genes. The complete array data will be reported elsewhere (L. Marselli, D. C. Sgroi, J. Thorne, S. Dahiya, A. Sharma, S. Bonner-Weir, P. Marchetti and G.C. Weir, unpublished results).

Pancreatic islet preparation and culture With the approval of the Ethics Committee in Pisa, Italy, human islets were isolated from 12 ND donors (age 58±6 years, seven women/five men, BMI 24.1±0.7 kg/m^2) by collagenase digestion and density gradient purification [9], cultured in M199 medium (5.5 mmol/l glucose) and shipped to Brussels, Belgium, within 1–5 days of isolation. After overnight recovery in Ham's F-10 containing 6.1 mmol/l glucose, 10% (vol./vol.) FCS, 2 mmol/l GlutaMAX (Gibco, Invitrogen, Merelbeke, Belgium), 50 μmol/l 3-isobutyl-1-methylxanthine, 1% (wt/vol.) charcoal-absorbed BSA (Boehringer, Indianapolis, IN, USA), 50 U/ml penicillin and 50 μg/ml streptomycin, islets were exposed to NEFA in the same medium without FCS and a glucose concentration of 6.1 or 28 mmol/l [10]. The percentage of beta cells, examined by insulin immunofluorescence (see below), in the islet preparations was 50±3% (range 35% to 70%, n=12).

Oleate and palmitate (sodium salt, Sigma, Bornem, Belgium) were dissolved in 90% (vol./vol.) ethanol and diluted 1:100 to a final concentration of 0.5 mmol/l, corresponding to a NEFA/BSA ratio of 3.4 [10, 26]. The control condition contained 1% charcoal-absorbed BSA and a similar ethanol dilution. IL-1 receptor antagonist (IL-1ra; R&D Systems, Abingdon, UK) was used alone, or in combination with palmitate, at a concentration of 30 ng/ml. In combination with recombinant human IL-1β (50 U/ml) and IFN-γ (1,000 U/ml; PeproTech, Paris, France), 300 ng/ml IL-1ra was used (>100-fold concentration compared with the added IL-1β) and added 30 min prior to cytokines [27]. Recombinant murine TNF-α (Innogenetics, Gent, Belgium) was used at a concentration of 1000 U/ml. Cyclopiazonic acid (CPA; Sigma) was used at 50 μmol/l [10] and salubrinal (ChemBridge, San Diego, CA, USA) at 75 μmol/l [28].

Human ductal cell lines Pancreatic ductal adenocarcinoma, Capan-2, and epithelioid carcinoma, Panc-1, cell lines [29] were a kind gift of L. Bouwens (Vrije Universiteit Brussel, Brussels, Belgium). Capan-2 cells were cultured in Advanced-RPMI medium (Gibco, Paisley, UK) containing 10% (vol./vol.) FCS, 2 mmol/l GlutaMAX and antibiotics (as above). Panc-1 cells were cultured in DMEM (without pyruvate; Gibco) containing 10% (vol./vol.) serum, MEM non-essential amino acids (Sigma) and antibiotics. For the NEFA treatment, medium contained 1% (vol./vol.) FCS and 1% (wt/vol.) BSA.

Assessment of cell death The percentage of islet cell death was determined by at least two observers (one unaware of sample identity) using inverted fluorescence microscopy following staining with propidium iodide (5 μg/ml; Sigma) and Hoechst 33342 (10 μg/ml; Sigma) as described [10, 30].

Real-time PCR Poly(A)$^+$ RNA was isolated from human islets and reverse transcribed. The real-time PCR amplification was done using iQ SyBR Green Supermix on iCycler MyiQ Single Color (BIO-RAD, Hercules, CA, USA) as described [10]. The gene expression level was normalised to the housekeeping gene β-actin, expression of which is not modified by the treatments and comparable with that of ornithine decarboxylase antizyme 1 (*OAZ1*; ESM Fig. 1). Primer sequences are given in ESM Tables 2 and 3.

Immunofluorescence Dispersed human islet cells were stained with anti-p65 (Santa Cruz Biotechnology, Santa

Cruz, CA, USA) and anti-insulin antibodies (Sigma) as described in the ESM. To determine the islet cell type(s) producing chemokine (C-C motif) ligand (CCL)2 pancreatic specimens were stained by double immunofluorescence using anti-CCL2 (Abcam, Cambridge Sciences Park, Cambridge, UK), anti-insulin (Dako, Glostrup, Denmark), and anti-glucagon (R&D Systems) antibodies and analysed with a Leica TCS SP5 laser scanning confocal microscope (see ESM).

Insulin RIA Insulin accumulated in the culture medium was measured as described [30] and normalised to β-actin mRNA expression.

Chemokine/cytokine ELISA Human chemokine (C-X-C motif) ligand 1 (CXCL1) and IL-6 protein were measured in the culture medium by ELISA following the manufacturer's instructions (Quantikine, R&D Systems, Minneapolis, MN, USA) and normalised to β-actin mRNA expression.

Statistical analysis Data are means±SEM. Real-time PCR data were log transformed to obtain normal distribution and compared by ANOVA followed by paired *t* test with the Bonferroni correction for multiple comparisons. Microarray data were analysed by Mann–Whitney test. Linear regression was done using the least squares method. Multiple regression models were built to determine whether the association between a dependent and an independent variable remained significant after adjusting for other independent variables. A p value<0.05 was considered significant.

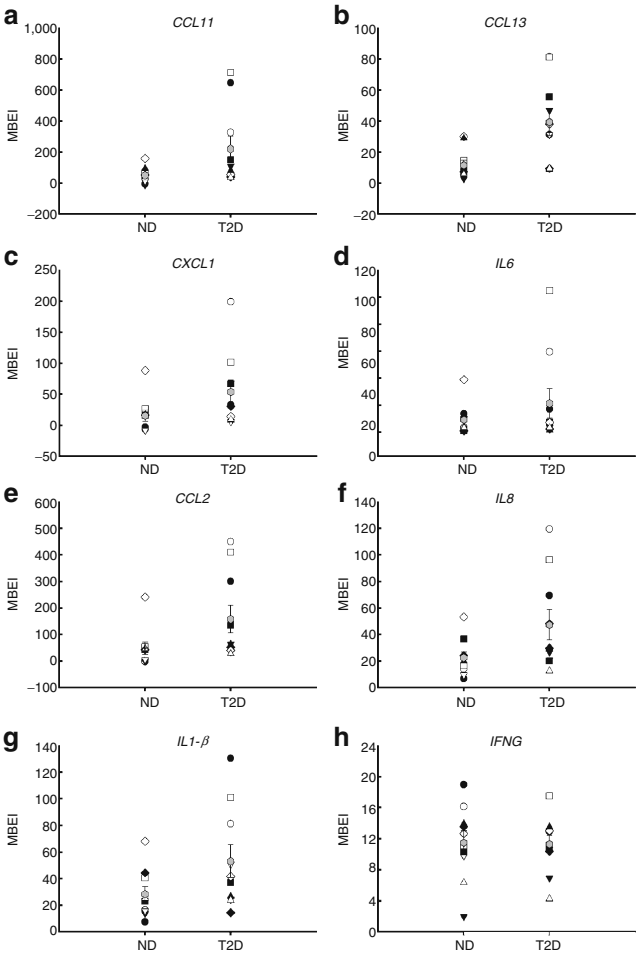

Fig. 1 Expression of pro-inflammatory chemokines and cytokines in human beta cells from type 2 diabetic patients and ND controls. Microarray analysis of LCM human beta cells from ten ND and ten type 2 diabetic (T2D) donors. Individual mRNA levels (expressed as model based expression indices, MBEI) and means±SEM are shown in black/white and grey symbols, respectively. **a** *CCL11*, p=0.063; **b** *CCL13*, p=0.001; **c** *CXCL1*, p=0.089; **d** *IL6*, p=0.48; **e** *CCL2*, p=0.015; **f** *IL8*, p=0.063; **g** *IL1-β*, p=0.105; **h** *IFNG*, p=0.91

Results

Microarray profiling of human beta cells reveals increased cytokine and chemokine expression in type 2 diabetes Human beta cells obtained from type 2 diabetic donors by laser-capture microdissection had increased mRNA expression of chemokines and cytokines compared with ND controls. The expression of *CCL2* (also known as *MCP-1*) and *CCL13* (also known as *MCP-4*) was threefold higher in type 2 diabetic beta cells (p<0.05, Fig. 1), while *CCL11* (eotaxin), *CXCL1* (Gro-α) and *IL6* expression tended to be higher (Fig. 1).

Array data on *IL1-β* (also known as *IL1B*) and *IL8* from our LCM samples has already been reported and confirmed by real-time PCR [20]. These previously published data were obtained for nine ND and ten type 2 diabetic patients; one of the ten controls with no type 2 diabetes history was excluded because of some high glycaemia readings during the antemortem period. Values were obtained from an initial analysis in which filtering criteria were used and the signals and statistical results of 30,990 probe sets were retrieved. The published results for *IL1-β* were 28±3.9 vs 58±14.2 (p=0.032) and for *IL8* 22.2±3.8 vs 50.7±12.9 (p=0.034) for ND vs type 2 diabetic patients, respectively [20]; for all ten ND, *IL1-β* was 27±3.7 (p=0.057 vs type 2 diabetes) and *IL8* 22.2±3.8 (p=0.059).

For our present study of ten ND controls and ten type 2 diabetic patients, analysis was performed to obtain the expression signal and the statistical results of all the probe sets present in the chip, returning a list of 61,295 probes, and no individuals were excluded. With this approach, data for *IL1-β* were 28.3±5.7 vs 53.2±12.2 and for *IL8* 22.7±4.3 vs 47.3±11.4 (Fig. 1). Interferon-γ (*IFNG*) expression was similar in type 2 diabetes and controls. The cytokine and

chemokine expression in type 2 diabetic beta cells varied considerably between individuals, suggesting that an inflammatory response was present in islets from some but not all donors. The presence of CCL2 protein was detected in insulin-positive beta cells in pancreatic tissue sections from ND and type 2 diabetic donors using confocal microscopy, whereas little or no CCL2 was detected in glucagon-positive alpha cells or exocrine cells (Fig. 2).

Palmitate, but not oleate or high glucose, induces expression of chemokines and cytokines in human islets We next examined whether metabolic stress plays a role in islet inflammation. The mild inflammatory response observed in type 2 diabetic beta cells was mimicked in vitro by ND human islet exposure to palmitate. Palmitate increased the mRNA expression of *CXCL1*, *IL6*, *CCL2*, *IL8*, *IL1-β* and *TNF-α* (also known as *TNF*) by two- to tenfold after 24 h and 48 h (Fig. 3). In contrast, oleate did not induce any of the chemokines and cytokines (Fig. 3). The mRNA induction by palmitate was paralleled by protein secretion by the human islets. Palmitate induced secretion of IL-6 and CXCL1 by two- to threefold after 24 and 48 h (Fig. 4). The induction of cytokines by palmitate was paralleled by the upregulation of nuclear factor κB inhibitor, α (IκBα; Fig. 3), a direct target of the transcription factor NF-κB. At variance with a previous report describing IL-1β induction in human islets cultured for 44 h at 33 mmol/l glucose [21], 28 mmol/l glucose neither induced chemokine/cytokine mRNA/protein production nor IκBα expression (Figs 3 and 4), while it did increase insulin release to the culture medium (Fig. 4). This is in line with our previous report on high-glucose-treated human islets [23]. Moreover, high glucose did not potentiate the effects of palmitate (Figs 3 and 4). We have previously shown that neither NEFA nor high glucose induces chemokines or cytokines in rodent insulin-secreting cells [7], suggesting that the observed pro-inflammatory effects of palmitate are present in human but not in rodent islets of Langerhans.

Human islet chemokine and cytokine expression is positively correlated with IL-1β and TNF-α The expression of *IL8*, *IL6*, *CXCL1* and *CCL2* in the cultured human islet samples was positively correlated with the expression of *IL1-β* and *TNF-α* (Fig. 5). To examine whether an independent association exists between *IL1-β*, *TNF-α* and *IL6* mRNA (considered as independent variables) and downstream chemokines and cytokines, *CXCL1*, *CCL2* and *IL8* mRNA (considered as dependent variables), multiple regression analysis was performed. *IL1-β* and *TNF-α* but not *IL6* were significantly associated with *CXCL1* and *CCL2* ($p<0.01$), while all three cytokines were independently correlated with *IL8* expression ($p<0.01$).

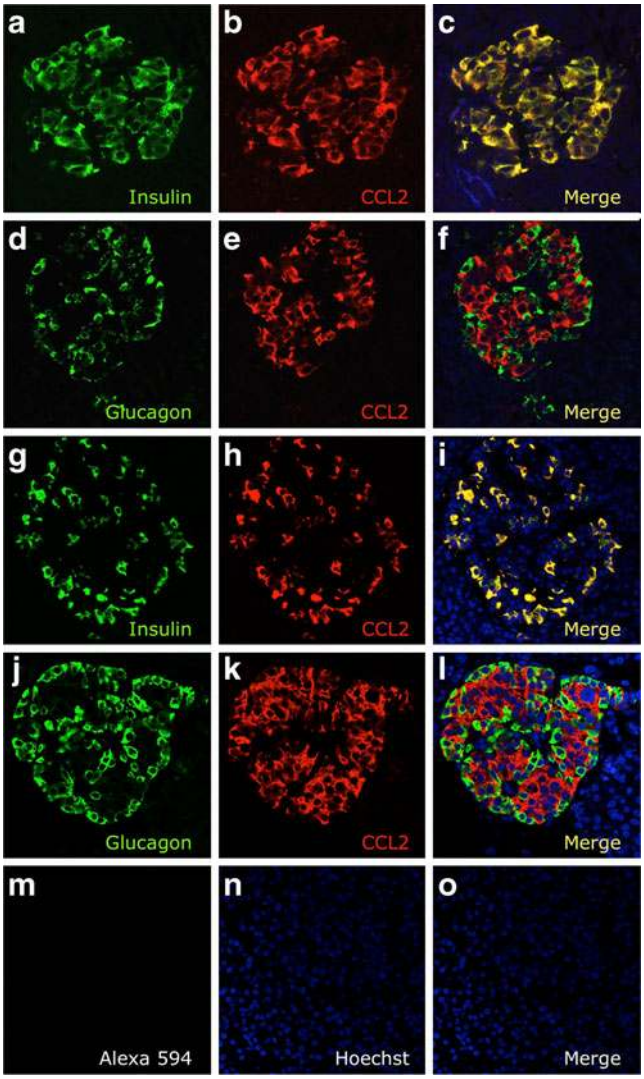

Fig. 2 CCL2 co-localises with insulin in human islets. Confocal microscopy analysis of insulin- (**a**, **g**) and CCL2-stained (**b**, **h**) sections of pancreas from ND donors (**a–c**) and type 2 diabetic patients (**g–i**) shows predominant presence of CCL2 protein in insulin-positive cells. In the merged images (**c**, **i**), the superposition of insulin (green) and CCL2 (red) results in a yellow colour, while Hoechst 33342 stains nuclei blue. In contrast, in glucagon- **(d**, **j)** and CCL2-stained (**e, k**) sections of pancreas from ND donors (**d–f**) and type 2 diabetic patients (**j–l**) there is little or no CCL2 protein in glucagon-positive cells. In the merged images (**f**, **l**), there is no yellow colour seen when glucagon (green) and CCL2 (red) are superposed. Images are representative of 85 islets examined in pancreases from three ND donors and 60 islets in pancreas samples from two type 2 diabetic patients. The lower panels (**m–o**) show negative control sections probed with anti-rabbit Alexa 594 in the absence of primary antibody: **m** Alexa 594; **n** Hoechst; **o** merged

IL-1β mediates the chemokine/cytokine induction by palmitate To establish whether IL-1β is upstream and causal in the inflammatory response induced by palmitate in human islets, we blocked IL-1β signalling using IL-1ra as previously described [27]. As a positive control for IL-1ra activity, we used it in combination with the cytokines IL-1β

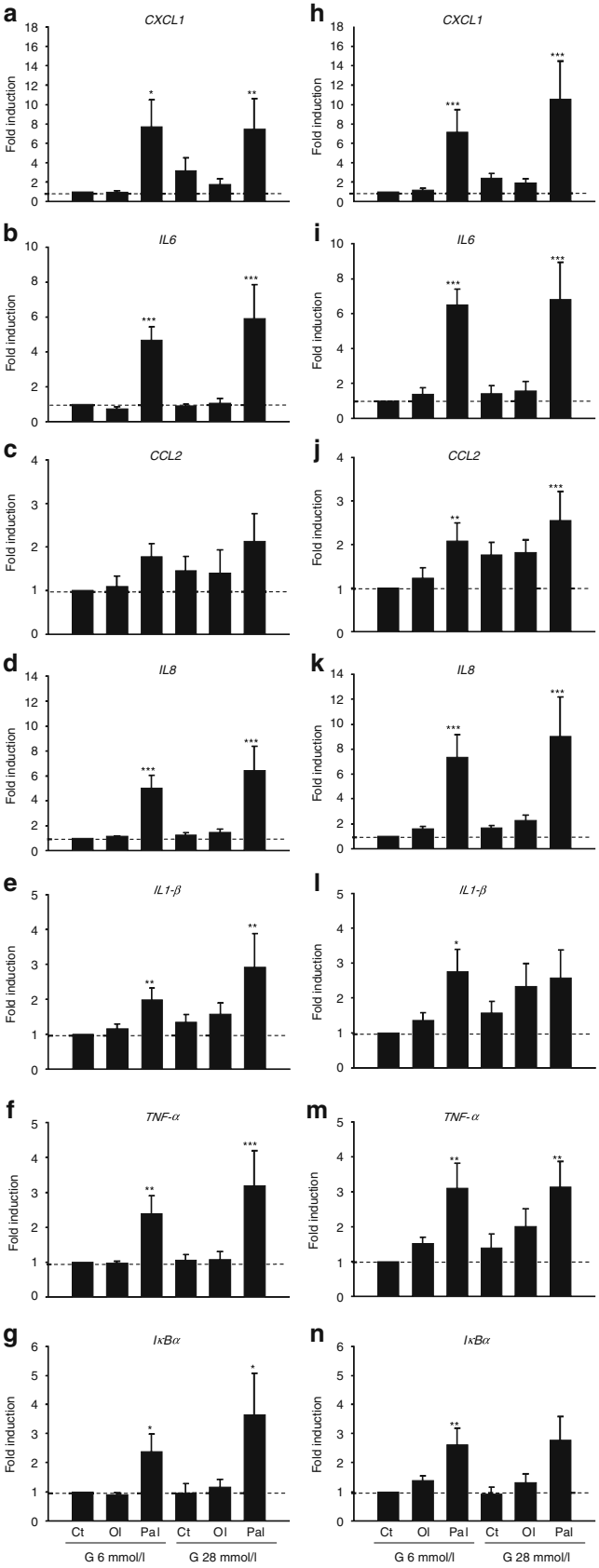

Fig. 3 Palmitate, but not oleate or high glucose, induces chemokine and cytokine mRNA expression in human islets. Human islets from ND donors were cultured for 24 h (**a–g**) or 48 h (**h–n**) in the absence (Ct) or presence of oleate (Ol) or palmitate (Pal) at 6 or 28 mmol/l glucose (G). Expression of mRNA was measured by real-time PCR, corrected by β-actin mRNA and expressed as fold induction compared with control (Ct, 6 mmol/l glucose). **a**, **h** *CXCL1*; **b**, **i** *IL6*; **c**, **j** *CCL2*; **d**, **k** *IL8*; **e**, **l** *IL1-β*; **f**, **m** *TNF-α*; **g, n** *IκBα*. The results are means ± SEM of six to nine independent experiments. *$p<0.05$; **$p<0.01$; ***$p<0.001$ vs Ct at glucose 6 mmol/l

and IFN-γ. IL-1β and IFN-γ induced a much greater expression of the mRNA of cytokines and chemokines (three- to tenfold, Fig. 6) than palmitate. IL-1ra significantly inhibited the cytokine-induced expression of *IL6* at the mRNA level and IL-6 production at the protein level (by seven- and ninefold, Fig. 6) and also decreased expression of the other chemokines and cytokines (Fig. 6). Interestingly, IL-1ra prevented palmitate-induced *IL6* mRNA expression and IL-6 secretion, as well as the induction of *CXCL1*, *IL8* and *IκBα* (also known as *NFKBIA*; Fig. 6). IL-1ra also decreased basal expression of *IL6*, *CXCL1*, *CCL2*, *IL8* and *TNF-α*, pointing to a basal IL-1β-induced

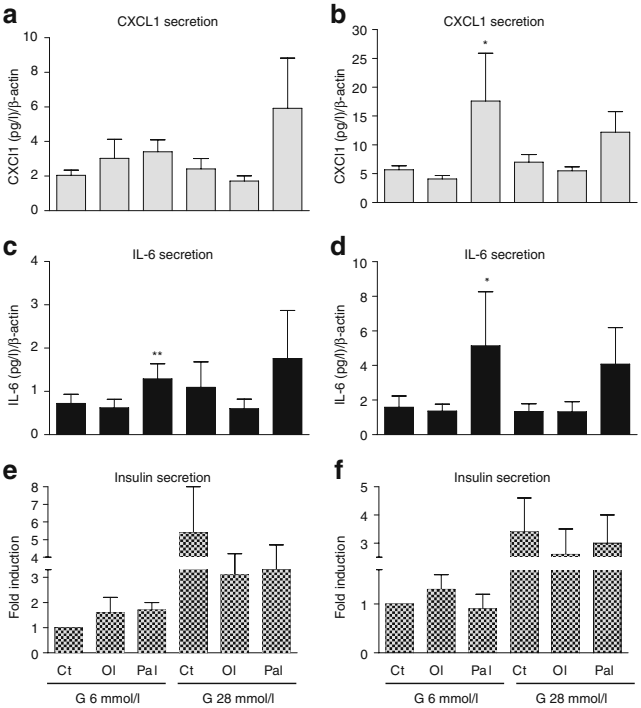

Fig. 4 Palmitate, but not oleate or high glucose, induces IL-6 and CXCL1 protein secretion by human islets. Human islets were cultured for 24 h (**a**, **c**, **e**) or 48 h (**b**, **d**, **f**) in the absence (Ct) or presence of oleate (Ol) or palmitate (Pal) at 6 or 28 mmol/l glucose (G). The chemokine and cytokine secreted to the culture medium were measured by ELISA, and values are expressed in pg/l normalised to β-actin mRNA expression. Insulin released to the medium was measured by RIA and normalised to β-actin mRNA. **a**, **b** CXCL1; **c**, **d** IL-6; **e**, **f** insulin secretion. The results are means±SEM of four to six independent experiments. *$p<0.05$; **$p<0.01$ vs Ct at G 6 mmol/l

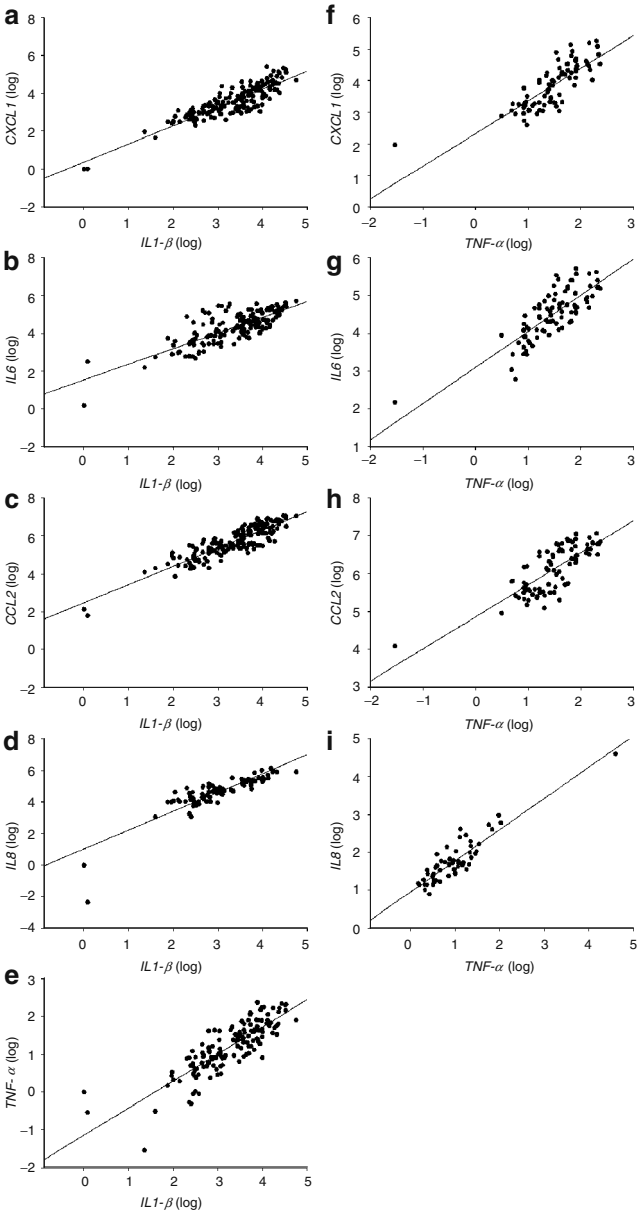

Fig. 5 Expression of chemokine and cytokine mRNA is positively correlated with *IL1-β* and *TNF-α* mRNA levels in cultured human islets. Linear correlation between log-transformed chemokine and cytokine mRNA and *IL1-β* (**a–e**) and *TNF-α* (**f–i**) mRNA expression in human islets cultured under different glucose and NEFA conditions for 24 h and 48 h; n=135 samples. Correlation of *IL1-β* with: **a** *CXCL1*, R=0.85, p<0.001; **b** *IL6*, R=0.75, p<0.001; **c** *CCL2*, R=0.87, p<0.001; **d** *IL8* R=0.94, p<0.001; **e** *TNF-α*, R=0.85, p<0.001. Correlation of *TNF-α* with: **f** *CXCL1*, R=0.81, p<0.001; **g** *IL6*, R=0.81, p<0.001; **h** *CCL2*, R=0.78, p<0.001; **i** *IL8* R=0.91, p<0.001

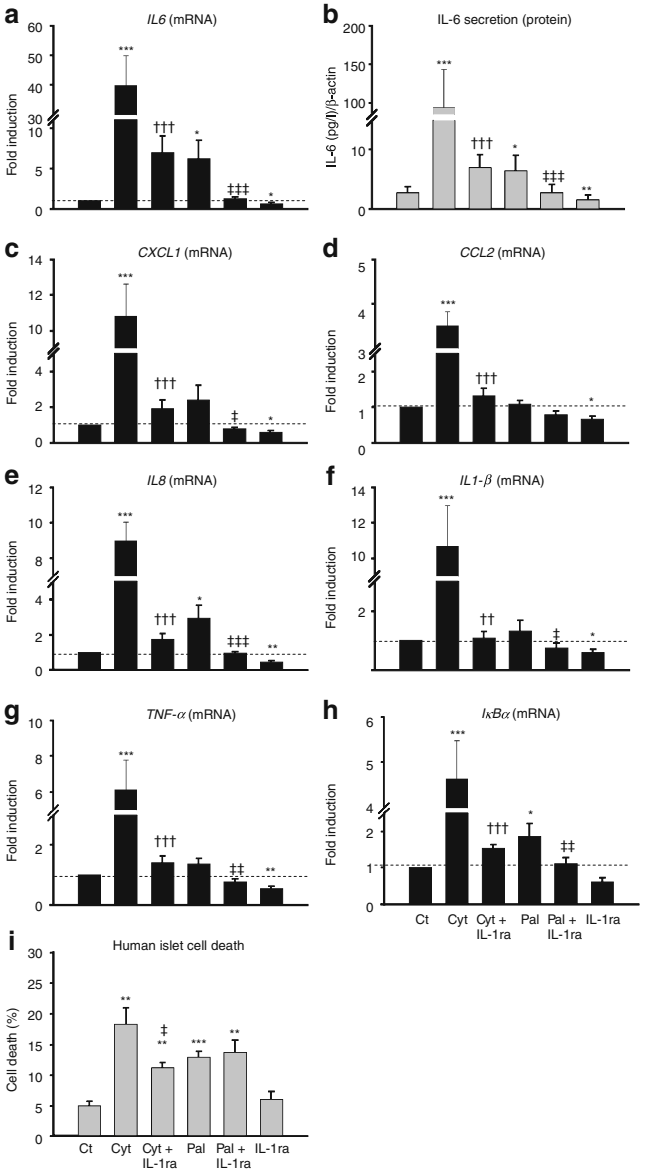

Fig. 6 IL-1ra prevents palmitate-induced chemokine/cytokine mRNA expression and protein production in human islets but not islet cell death. For mRNA and protein analysis, human islets were cultured for 48 h in the presence of palmitate (Pal), alone or in combination with IL-1ra. The cytokines (Cyt) IL-1β (50 U/ml) plus IFN-γ (1,000 U/ml), alone or in combination with IL-1ra, were used as positive controls. Chemokine, cytokine and *IκBα* mRNA expression was analysed by real-time PCR and IL-6 protein by ELISA. mRNA data are expressed as fold induction of control (Ct) and IL-6 secretion as pg/l normalised to β-actin. Results are means±SEM of eight independent experiments. For the evaluation of human islet cell death, islets were cultured for 3 days. **a** *IL6* mRNA expression; **b** IL-6 protein secretion; **c** *CXCL1* mRNA expression; **d** *CCL2* mRNA expression; **e** *IL8* mRNA expression; **f** *IL1-β* mRNA expression; **g** *TNF-α* mRNA expression; **h** *IκBα* mRNA expression; **i** human islet cell death. The results are means±SEM of four independent experiments. *p<0.05, **p<0.01; ***p<0.001 for treated vs control; $^{\dagger}p$<0.05, †† p<0.01; $^{\dagger\dagger\dagger}p$<0.001 for cytokine vs cytokine+IL-1ra; $^{\ddagger}p$<0.05, $^{\ddagger\ddagger}p$<0.01; $^{\ddagger\ddagger\ddagger}p$<0.001 for palmitate vs palmitate + IL-1ra

chemokine production by human islets, as previously reported [23]. The palmitate-induced *TNF-α* expression was also prevented by IL-1ra, suggesting that it is downstream of IL-1β signalling. While IL-1ra reduced cytokine-induced human islet cell death, blocking IL-1β signalling did not protect against palmitate (Fig. 6),

suggesting that *IL1-β* induction by palmitate regulates the expression of downstream cytokines and chemokines, but does not play a major role in lipotoxic cell death.

Cell source of the chemokines and cytokines produced by human islets Because the purification of human beta cells from islet preparations is technically challenging, we evaluated the cell type in which palmitate induces NF-κB activation by staining dispersed human islet cells for insulin and p65, the major NF-κB component in beta cells. Palmitate, and the combination of IL-1β plus IFN-γ plus TNF-α used as positive control, induced NF-κB activation in beta and non-beta cells, indicated by nuclear translocation of p65 after 2 h (ESM Fig. 2). Palmitate also activated NF-κB in the clonal human ductal cells Panc-1 and Capan-2 (ESM Fig. 3), and it induced chemokines and cytokines in ductal cells, while oleate did not (ESM Fig. 3). This suggests that both human beta cells and exocrine ductal cells exhibit pro-inflammatory responses to saturated NEFA.

Role of ER stress in the induction of chemokines and cytokines by palmitate ER stress is present in islets from type 2 diabetic individuals [31], and can be elicited in human islets in vitro by saturated NEFA [10]. To examine whether ER stress signalling contributes to the observed induction of chemokines and cytokines, we exposed human islets to the synthetic ER stressor CPA. This ER stressor induced cytokine expression and NF-κB activation to a similar extent as palmitate (ESM Fig. 4). To evaluate the role of the PERK/eukaryotic translation initiation factor 2, subunit 1 α (eIF2α) pathway on chemokine/cytokine expression we used salubrinal, a selective inhibitor of eIF2α dephosphorylation [28, 30]. Salubrinal did not lead to chemokine/cytokine or IκBα expression in human islets (ESM Fig. 5), suggesting that other branches of the ER stress response mediate the pro-inflammatory response.

Discussion

Systemic low-grade inflammation precedes the onset of type 2 diabetes by years [14], but its role in the pathogenesis of the disease is debated. In genetic studies, a polymorphism in *CCL2* has been associated with type 2 diabetes risk [32]. In a meta-analysis of type 2 diabetes genome-wide association scans, however, gene variants influencing circulating levels of IL-1ra, IL-18, the IL-6 receptor, macrophage migration inhibitory factor and C-reactive protein did not predispose to type 2 diabetes [33], suggesting that inflammation might be secondary to rather than causal for diabetes. Human and animal studies point to a role for inflammation in enlarged adipose tissues, increased secretion of cytokines and chemokines by adipocytes and fat-infiltrating macrophages, and insulin resistance [14, 15]. Whether inflammation is also present in islets and whether this contributes to the development of type 2 diabetes are important questions because of the central role of beta cell dysfunction in the disease pathogenesis [22]. Moreover, IL-1ra therapy is under evaluation for the treatment of type 2 diabetes [34, 35], based on studies suggesting that high-glucose-induced IL-1β secretion by beta cells causes beta cell dysfunction and death [21].

In the present study, we observed a gene expression signature of mild inflammation in human type 2 diabetic beta cells obtained by LCM. There was increased expression of the mRNA for: the β-chemokines CCL2 and CCL13, implicated in monocyte chemotaxis [15]; α-chemokines CXCL1 and IL-8, potent chemoattractants and activators of neutrophils [15]; and the pro-inflammatory cytokines IL-1β and IL-6. The chemokine and cytokine mRNA expression was heterogeneous in the type 2 diabetic group, suggesting that mild inflammation is present in some but not all type 2 diabetic individuals. Mild macrophage infiltration has been observed in islets from type 2 diabetic individuals in two recent studies, with 20% of the islets containing >3 $CD68^+$ cells per islet compared with 5% of the ND islets [18, 19]. This islet inflammation could be mediated by CCL2 and CCL13 produced in human beta cells. Islet inflammation was also present in animal models for type 2 diabetes, namely the high-fat-diet-fed and *db*/*db* mice [18, 36], the Cohen diabetic rat [36] and the Goto–Kakizaki rat [37, 38]. The role of these infiltrating macrophages is unknown. Some propose them to be beneficial to islet function by promoting angiogenesis [39]; others suggest that activated $CD68^+$ cells could cause beta cell dysfunction and death [36, 37]. The beta cell inflammatory response was presently reproduced at the RNA and protein level in ND human islets by their in vitro exposure to the saturated NEFA palmitate, and was dependent on the canonical mediator of early inflammation IL-1β. While confocal microscopy suggests that CCL2 protein production in ND and type 2 diabetic pancreas is largely restricted to beta cells, palmitate induced a pro-inflammatory response in both beta and islet non-beta and ductal cells.

At variance with previous reports that elevated glucose concentrations increase *IL1-β* expression in human islets cultured either in suspension or on an extracellular matrix [20, 21], glucose did not induce an inflammatory response in human islets in this or a previous study by our group [23]. Rather, the chemokine/cytokine expression and NF-κB activation was induced by the saturated NEFA palmitate, but not by the unsaturated oleate. Saturated and unsaturated NEFA have different functional effects in beta cells [40–42]. Saturated NEFA are pro-inflammatory in several cell types

[43, 44] and also cause ER stress [10]. Because palmitate (but not oleate or high glucose) induces ER stress in human islets [10] and there is a crosstalk between ER stress and NF-κB pathways [45–47], we explored this mechanism further. We have previously shown that palmitate activates PERK, and also leads to ATF6 and IRE1 activation through depletion of ER Ca^{2+} stores [10]. The crosstalk between ER stress and NF-κB activation can occur at different levels. Ca^{2+} efflux from the ER activates NF-κB through generation of reactive oxygen species [46] and release of a diffusible, still unknown, NF-κB activator [48]. IRE1 and TNF receptor-associated factor 2 (TRAF-2) can activate NF-κB [45], and stalled protein translation caused by eIF2α phosphorylation decreases IκBα protein availability, consequently activating NF-κB [49]. CPA, a canonical ER stressor, upregulates chemokines and cytokines and activates NF-κB in human islets with an effect comparable with that of palmitate (present data). The CPA-induced p65 nuclear translocation in beta and non-beta cells occurred after short (2 h) exposure, in line with previously reported kinetics showing maximal NF-κB activation by CPA and thapsigargin after 1 h due to ER Ca^{2+} depletion [50]. To examine involvement of the PERK pathway in NF-κB activation, we used salubrinal as a selective inhibitor of eIF2α dephosphorylation [28, 30]. Salubrinal did not induce chemokines/cytokines or IκBα (ESM Fig. 5), suggesting that ER Ca^{2+} depletion or ER stress-induced IRE1 signalling but not inhibition of protein translation mediates palmitate-induced NF-κB activation in human islets. ER stress is not the only mechanism by which palmitate could induce mild inflammation in human islets. Toll-like receptors (TLRs) may also recognise saturated NEFA [51]. These TLRs have been suggested to mediate some of the NEFA-induced islet inflammation in a recent study [52].

Blocking IL-1β signalling with IL-1ra prevented the palmitate-induced expression of chemokines and cytokines and NF-κB activation, showing that the observed inflammatory response is largely dependent on IL-1β production. The magnitude of IL-1β induction is modest, and probably insufficient to cause human islet cell damage. In fact, low concentrations of IL-1β have been shown to stimulate human islet function [53]. IL-1ra did not prevent palmitate-induced human islet cell death in vitro, suggesting that the mild IL-1β induction is not directly deleterious to the human islets and does not mediate lipotoxicity. Administration of IL-1ra in vivo to high-fat-fed mice improved beta cell function but also fully protected against changes in insulin sensitivity [54]. IL-1ra reduced adipose tissue inflammation, altered adipokine secretion and decreased circulating lipid levels, which may have contributed to the preservation of insulin secretion and glucose tolerance in the high-fat-fed mice. We propose, therefore, that the induction of IL-1β and downstream chemokines and cytokines by saturated NEFA results in mild islet inflammation in type 2 diabetes, but that this does not directly contribute to beta cell dysfunction and apoptosis. ER stress [31] and other signalling pathways [55] contribute to lipotoxicity in human islets in type 2 diabetes.

In conclusion, we demonstrate here that there is a mild inflammatory process in islets from some but not all type 2 diabetic patients. Palmitate, but not oleate or high glucose, induces in vitro chemokine and cytokine expression in ND human islets, a process largely dependent on IL-1β production. ER stress could be one of the mechanisms involved in saturated-fatty-acid-induced human islet inflammation. The induction of IL-1β and downstream chemokines and cytokines by saturated NEFA does not directly contribute to lipotoxicity.

The mild inflammation in islets from type 2 diabetic patients could be the result of in vivo exposure to saturated NEFA. Whether this islet inflammation causes loss of functional beta cell mass and thereby contributes to the development and progression of type 2 diabetes remains to be elucidated.

Acknowledgements We thank G. Vandenbroeck, M. Urbain, J. Schoonheydt and R. Leeman (Laboratory of Experimental Medicine) for expert technical assistance. This work was supported by the European Union (Integrated Project EuroDia in the Framework Programme [FP] 6 and Collaborative Projects CEED3 and NAIMIT in FP7); the Belgian Program on Interuniversity Poles of Attraction (IUAP P6/40); the Fonds National de la Recherche Scientifique (FNRS), Fonds de la Recherche Scientifique Médicale (FRSM) and Actions de Recherche Concertées de la Communauté Française, Belgium (M. Cnop and D. L. Eizirik); the Italian Diabetes Research Foundation-FORISID and the Italian Ministry of Health (F. Dotta); the European Association for the Study of Diabetes–European Foundation for the Study of Diabetes GlaxoSmithKline research grant (L. Marselli).

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

References

1. Weyer C, Bogardus C, Mott DM, Pratley RE (1999) The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus. J Clin Invest 104:787–794
2. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC (2003) β-cell deficit and increased β-cell apoptosis in humans with type 2 diabetes. Diabetes 52:102–110
3. Bindokas VP, Kuznetsov A, Sreenan S, Polonsky KS, Roe MW, Philipson LH (2003) Visualizing superoxide production in normal and diabetic rat islets of Langerhans. J Biol Chem 278:9796–9801
4. Kaneto H, Xu G, Fujii N, Kim S, Bonner-Weir S, Weir GC (2002) Involvement of c-Jun N-terminal kinase in oxidative stress-mediated suppression of insulin gene expression. J Biol Chem 277:30010–30018
5. Piro S, Anello M, Di Pietro C et al (2002) Chronic exposure to free fatty acids or high glucose induces apoptosis in rat pancreatic islets: possible role of oxidative stress. Metabolism 51:1340–1347

6. Kelpe CL, Moore PC, Parazzoli SD, Wicksteed B, Rhodes CJ, Poitout V (2003) Palmitate inhibition of insulin gene expression is mediated at the transcriptional level via ceramide synthesis. J Biol Chem 278:30015–30021
7. Kharroubi I, Ladriere L, Cardozo AK, Dogusan Z, Cnop M, Eizirik DL (2004) Free fatty acids and cytokines induce pancreatic β-cell apoptosis by different mechanisms: role of nuclear factor-κB and endoplasmic reticulum stress. Endocrinology 145:5087–5096
8. Laybutt DR, Preston AM, Akerfeldt MC et al (2007) Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes. Diabetologia 50:752–763
9. Lupi R, Dotta F, Marselli L et al (2002) Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that β-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated. Diabetes 51:1437–1442
10. Cunha DA, Hekerman P, Ladriere L et al (2008) Initiation and execution of lipotoxic ER stress in pancreatic β-cells. J Cell Sci 121:2308–2318
11. Sakuraba H, Mizukami H, Yagihashi N, Wada R, Hanyu C, Yagihashi S (2002) Reduced β-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese type II diabetic patients. Diabetologia 45:85–96
12. Huang CJ, Lin CY, Haataja L et al (2007) High expression rates of human islet amyloid polypeptide induce endoplasmic reticulum stress mediated β-cell apoptosis, a characteristic of humans with type 2 but not type 1 diabetes. Diabetes 56:2016–2027
13. Marchetti P, Bugliani M, Lupi R et al (2007) The endoplasmic reticulum in pancreatic beta cells of type 2 diabetes patients. Diabetologia 50:2486–2494
14. Dandona P, Aljada A, Bandyopadhyay A (2004) Inflammation: the link between insulin resistance, obesity and diabetes. Trends Immunol 25:4–7
15. Kolb H, Mandrup-Poulsen T (2005) An immune origin of type 2 diabetes? Diabetologia 48:1038–1050
16. Spranger J, Kroke A, Mohlig M et al (2003) Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. Diabetes 52:812–817
17. Donath MY, Storling J, Maedler K, Mandrup-Poulsen T (2003) Inflammatory mediators and islet β-cell failure: a link between type 1 and type 2 diabetes. J Mol Med 81:455–470
18. Ehses JA, Perren A, Eppler E et al (2007) Increased number of islet-associated macrophages in type 2 diabetes. Diabetes 56:2356–2370
19. Richardson SJ, Willcox A, Bone AJ, Foulis AK, Morgan NG (2009) Islet-associated macrophages in type 2 diabetes. Diabetologia 52:1686–1688
20. Boni-Schnetzler M, Thorne J, Parnaud G et al (2008) Increased interleukin (IL)-1β messenger ribonucleic acid expression in β-cells of individuals with type 2 diabetes and regulation of IL-1β in human islets by glucose and autostimulation. J Clin Endocrinol Metab 93:4065–4074
21. Maedler K, Sergeev P, Ris F et al (2002) Glucose-induced β cell production of IL-1β contributes to glucotoxicity in human pancreatic islets. J Clin Invest 110:851–860
22. Cnop M, Welsh N, Jonas JC, Jörns A, Lenzen S, Eizirik DL (2005) Mechanisms of pancreatic β-cell death in type 1 and type 2 diabetes: many differences, few similarities. Diabetes 54(Suppl 2): S97–S107
23. Welsh N, Cnop M, Kharroubi I et al (2005) Is there a role for locally produced interleukin-1 in the deleterious effects of high glucose or the type 2 diabetes milieu to human pancreatic islets? Diabetes 54:3238–3244
24. Marselli L, Sgroi DC, Bonner-Weir S, Weir GC (2009) Laser capture microdissection of human pancreatic β-cells and RNA preparation for gene expression profiling. Methods Mol Biol 560:87–98
25. Marselli L, Thorne J, Ahn YB et al (2008) Gene expression of purified β-cell tissue obtained from human pancreas with laser capture microdissection. J Clin Endocrinol Metab 93:1046–1053
26. Cnop M, Hannaert JC, Hoorens A, Eizirik DL, Pipeleers DG (2001) Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. Diabetes 50:1771–1777
27. Eizirik DL, Tracey DE, Bendtzen K, Sandler S (1991) An interleukin-1 receptor antagonist protein protects insulin-producing β cells against suppressive effects of interleukin-1β. Diabetologia 34:445–448
28. Ladriere L, Igoillo-Esteve M, Cunha DA, et al. (2010) Enhanced signaling downstream of PERK potentiates lipotoxic endoplasmic reticulum stress in human islets. J Clin Endocrinol Metab 95:1442–1449
29. Sipos B, Moser S, Kalthoff H, Torok V, Lohr M, Kloppel G (2003) A comprehensive characterization of pancreatic ductal carcinoma cell lines: towards the establishment of an in vitro research platform. Virchows Arch 442:444–452
30. Cnop M, Ladriere L, Hekerman P et al (2007) Selective inhibition of eukaryotic translation initiation factor 2α dephosphorylation potentiates fatty acid-induced endoplasmic reticulum stress and causes pancreatic β-cell dysfunction and apoptosis. J Biol Chem 282:3989–3997
31. Eizirik DL, Cardozo AK, Cnop M (2008) The role for endoplasmic reticulum stress in diabetes mellitus. Endocr Rev 29:42–61
32. Simeoni E, Hoffmann MM, Winkelmann BR et al (2004) Association between the A-2518G polymorphism in the monocyte chemoattractant protein-1 gene and insulin resistance and type 2 diabetes mellitus. Diabetologia 47:1574–1580
33. Rafiq S, Melzer D, Weedon MN et al (2008) Gene variants influencing measures of inflammation or predisposing to autoimmune and inflammatory diseases are not associated with the risk of type 2 diabetes. Diabetologia 51:2205–2213
34. Larsen CM, Faulenbach M, Vaag A, Ehses JA, Donath MY, Mandrup-Poulsen T (2009) Sustained effects of interleukin-1 receptor antagonist treatment in type 2 diabetes. Diabetes Care 32:1663–1668
35. Larsen CM, Faulenbach M, Vaag A et al (2007) Interleukin-1-receptor antagonist in type 2 diabetes mellitus. N Engl J Med 356:1517–1526
36. Weksler-Zangen S, Raz I, Lenzen S et al (2008) Impaired glucose-stimulated insulin secretion is coupled with exocrine pancreatic lesions in the Cohen diabetic rat. Diabetes 57:279–287
37. Ehses JA, Lacraz G, Giroix MH et al (2009) IL-1 antagonism reduces hyperglycemia and tissue inflammation in the type 2 diabetic GK rat. Proc Natl Acad Sci USA 106:13998–14003
38. Homo-Delarche F, Calderari S, Irminger JC et al (2006) Islet inflammation and fibrosis in a spontaneous model of type 2 diabetes, the GK rat. Diabetes 55:1625–1633
39. Tessem JS, Jensen JN, Pelli H et al (2008) Critical roles for macrophages in islet angiogenesis and maintenance during pancreatic degeneration. Diabetes 57:1605–1617
40. Frigerio F, Brun T, Bartley C et al (2010) Peroxisome proliferator-activated receptor α (PPARα) protects against oleate-induced INS-1E beta cell dysfunction by preserving carbohydrate metabolism. Diabetologia 53:331–340
41. Maedler K, Spinas GA, Dyntar D, Moritz W, Kaiser N, Donath MY (2001) Distinct effects of saturated and monounsaturated fatty acids on β-cell turnover and function. Diabetes 50:69–76
42. Roche E, Buteau J, Aniento I, Reig JA, Soria B, Prentki M (1999) Palmitate and oleate induce the immediate-early response genes

c-fos and nur-77 in the pancreatic β-cell line INS-1. Diabetes 48:2007–2014
43. Ajuwon KM, Spurlock ME (2005) Palmitate activates the NF-κB transcription factor and induces IL-6 and TNFα expression in 3T3-L1 adipocytes. J Nutr 135:1841–1846
44. Staiger H, Staiger K, Stefan N et al (2004) Palmitate-induced interleukin-6 expression in human coronary artery endothelial cells. Diabetes 53:3209–3216
45. Kaneko M, Niinuma Y, Nomura Y (2003) Activation signal of nuclear factor-κB in response to endoplasmic reticulum stress is transduced via IRE1 and tumor necrosis factor receptor-associated factor 2. Biol Pharm Bull 26:931–935
46. Pahl HL, Baeuerle PA (1996) Activation of NF-κB by ER stress requires both Ca^{2+} and reactive oxygen intermediates as messengers. FEBS Lett 392:129–136
47. Tonnesen MF, Grunnet LG, Friberg J et al (2009) Inhibition of nuclear factor-κB or Bax prevents endoplasmic reticulum stress-but not nitric oxide-mediated apoptosis in INS-1E cells. Endocrinology 150:4094–4103
48. Glazner GW, Camandola S, Geiger JD, Mattson MP (2001) Endoplasmic reticulum D-myo-inositol 1,4,5-trisphosphate-sensitive stores regulate nuclear factor-κB binding activity in a calcium-independent manner. J Biol Chem 276:22461–22467
49. Jiang HY, Wek RC (2005) GCN2 phosphorylation of eIF2α activates NF-κB in response to UV irradiation. Biochem J 385:371–380
50. Pahl HL, Sester M, Burgert HG, Baeuerle PA (1996) Activation of transcription factor NF-κB by the adenovirus E3/19K protein requires its ER retention. J Cell Biol 132:511–522
51. Lee JY, Sohn KH, Rhee SH, Hwang D (2001) Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. J Biol Chem 276:16683–16689
52. Boni-Schnetzler M, Boller S, Debray S et al (2009) Free fatty acids induce a proinflammatory response in islets via the abundantly expressed interleukin-1 receptor I. Endocrinology 150:5218–5229
53. Eizirik DL, Welsh N, Hellerstrom C (1993) Predominance of stimulatory effects of interleukin-1β on isolated human pancreatic islets. J Clin Endocrinol Metab 76:399–403
54. Sauter NS, Schulthess FT, Galasso R, Castellani LW, Maedler K (2008) The antiinflammatory cytokine interleukin-1 receptor antagonist protects from high-fat diet-induced hyperglycemia. Endocrinology 149:2208–2218
55. Poitout V, Robertson RP (2008) Glucolipotoxicity: fuel excess and β-cell dysfunction. Endocr Rev 29:351–366