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Activation of 12-lipoxygenase in proinflammatory cytokine-mediated beta cell toxicity

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Abstract *Aims/hypothesis:* Beta cell inflammation and cytokine-induced toxicity are central to autoimmune diabetes development. Lipid mediators generated upon lipoxygenase (LO) activation can participate in inflammatory pathways. 12LO-deficient mice are resistant to streptozotocin-induced diabetes. This study sought to characterise the cellular processes involving 12LO-activation lipid inflammatory mediator production in cytokine-treated pancreatic beta cells. *Methods:* Islets and beta cell lines were treated with a combination of IL-1β, IFN-γ and TNF-α, or the 12LO product 12(*S*)-hydroxyeicosatetraenoic acid (HETE). Insulin secretion was measured using an enzyme immunoassay, and cell viability was evaluated using an in situ terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling assay. 12LO activity was evaluated and 12LO protein levels were determined using immunoblotting with a selective leucocyte type 12LO antibody. Cellular localisation of 12LO was evaluated using immunocytochemistry. *Results:* Basal expression of leucocyte type 12LO protein was found in human and mouse islets and in several rodent beta cell lines. In mouse β-TC3 cells, and in human islets, cytokines induced release of 12-HETE within 30 min. Cytokine addition also induced a rapid translocation of 12LO protein from the cytosol to the nucleus of β-TC3 cells as shown by subcellular fractionation and immunostaining. Cytokine-induced cell death and inhibition of insulin secretion were partially reversed by baicalein, a 12LO inhibitor. 12(*S*)-HETE inhibited β-TC3 cell insulin release in a time- and concentration-dependent manner. Incubating β-TC3 cells with 100 nmol/l of 12(*S*)-HETE resulted in a 57% reduction in basal insulin release (6 h), and a 17% increase in cell death (18 h) as compared with untreated cells. 12(*S*)-HETE activated the stress-activated protein kinase c-Jun N-terminal kinase and p38 within 15 min, as judged by increased kinase protein phosphorylation. *Conclusions/interpretation:* The data suggest that inflammatory cytokines rapidly activate 12LO and show for the first time that cytokines induce 12LO translocation. The effects of 12-HETE on insulin secretion, cytotoxicity and kinase activation were similar to the effects seen with cytokines. The results provide mechanistic information of cytokine-induced toxic effects on pancreatic beta cells and support the hypothesis that blocking 12LO activation could provide a new therapeutic way to protect pancreatic beta cells from autoimmune injury.

Keywords Apoptosis · Cytokines · Inflammation · 12-Lipoxygenase · Pancreatic beta cells · Type 1 diabetes mellitus

Abbreviations EIA: Enzyme immunoassay · ERK: Extracellular signal-regulated kinase · HETE: Hydroxyeicosatetraenoic acid · iNOS: Inducible nitric oxide synthase · JNK: c-Jun N-terminal kinase · 12LO: 12-Lipoxygenase · MAP kinase: Mitogen-activated protein kinase · MTT: 3-(4,5-dimethylthiazol-2-yl)2, 5-diphenyltetrazolium bromide · NOS: Nitric oxide synthase · STZ: Streptozotocin · TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling

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Introduction

12-Lipoxygenase (12LO) belongs to a class of mammalian dioxygenases that oxygenate polyunsaturated NEFA to generate eicosanoids including 12-hydroperoxyeicosatetraenoic acid and 12-hydroxyeicosatetraenoic acid (HETE) [1]. These lipid factors may be important in organism host defence and metabolic homeostasis. Abnormal production of lipids is also associated with pathological conditions [2–4], by accelerating inflammation, exerting local oxidative stress, and triggering and propagating intracellular

signalling cascades [5–7]. To date, three major isoforms of 12LO have been characterised in the mouse. They are platelet, leucocyte and epidermal 12LO with distinctive tissue distribution, substrate and product specificity. In contrast to platelet 12LO, leucocyte 12LO is widely distributed, including in the endocrine pancreas [8, 9].

12LO was initially thought to be physiologically important in regulating glucose-stimulated insulin release in pancreatic islets of Langerhans [10–13]. More recent evidence has suggested that 12LO plays a role in inflammation and metabolic-stress-induced islet functional abnormalities. 12LO message expression is induced under hyperglycaemic conditions in rat islets [14] along with cyclooxygenase. In diabetic Zucker Fatty rats, elevated 12LO expression is associated with deficits in insulin secretion and beta cell decompensation [15–17]. 12LO message, protein and activity can be induced by the proinflammatory cytokine IL-1β in rat islets and insulin-secreting beta cells [18]. Furthermore, a 12LO-null mouse demonstrates increased resistance to low-dose streptozotocin (STZ)-inflicted damage [19] as compared with wild-type control mice, implicating a role for 12LO in the development of type 1 diabetes. Another study shows that IL-1β, rather than affecting 12LO expression, increases its activity by providing the 12LO substrate, arachidonic acid, through activation of upstream calcium-independent phospholipase A_2 [20].

The mode of 12LO activation by proinflammatory cytokines and its biological effects on pancreatic beta cells are not completely understood. Studies of 12LO in other tissues have been informative. 12LO can be activated by various growth factors and cytokines in vascular smooth muscle cells [21]. LO products subsequently impose chemotactic, hypertrophic and mitogenic effects on vascular cells. 12LO is also a key enzyme controlling the cell cycle and inducing apoptosis in fibroblasts [22]. 12LO products enhance the interaction of monocytes with vascular endothelium, which initiates and accelerates atherogenesis, and they have been shown to participate in oxidative modification of lipoproteins and membrane lipids [3, 5, 23]. LO products could also increase intracellular oxidative stress by depleting glutathione and other reducing equivalents. Therefore, 12LO may have profound effects on cell metabolism and survival [24, 25]. In addition, 12LO products are recognised as intracellular signalling molecules capable of activating specific protein kinases [26, 27], translocating to specified cellular compartments [10], or modifying gene expression. Recent data also show that macrophages from the 12LO-null mouse lack IL-12 expression and production [28]. Thus, 12LO blockade could potentially modify TH1 cell development and reduce cell-mediated inflammation.

In this study, we evaluated the presence and regulation of leucocyte type 12LO in human and mouse islets and in islet cell lines. The biological effects of a major 12LO product, 12-HETE, on pancreatic beta cell function and viability were examined. The results support the possibility that 12LO activation plays an important role in cytokine-induced inflammatory effects in beta cells.

Materials and methods

Pancreatic islets and islet cell lines Human islets were from two sources. In some cases, islets were isolated from human pancreatic tissues following standard procedures in the islet core facility here at the University of Virginia [29]. The entire process was in compliance with the institutional Human Investigation Committee regulations (HIC# 8667). We were also supplied with high-quality human islets from the Human Islet Distribution Program supported by the Juvenile Diabetes Research Foundation. Mouse islets were isolated from C57B/L6 mice (Jackson Laboratory, Bar Harbor, ME, USA) in our laboratory, using enzyme digestion and density-gradient centrifugation [30]. Islets used to determine the presence of leucocyte 12LO were manually selected to achieve high purity. The studies were approved by the Institutional Animal Care and Use Committee at the University of Virginia.

Transformed mouse insulin-releasing β-TC3 cells were kindly provided by Dr Douglas Hanahan of the University of California at San Francisco. Cells from passage 30–50 were maintained in DMEM medium (Invitrogen, Rockville, MD, USA) containing 11 mmol/l glucose and supplemented with 10% heat-inactivated fetal BSA, 10 mmol/l HEPES, 200 μmol/l L-glutamine, 1 mmol/l sodium pyruvate, 50 units/ml penicillin and 50 μg/ml streptomycin at pH 7.4. The cells were cultured in a 37°C, humidified incubator supplied with 5% carbon dioxide. Fresh medium was replaced every 2 days. Unless otherwise stated, the cells were plated at a density of $10^5/cm^2$.

Cytokine, 12-HETE treatment and 12LO inhibition in β-TC3 cells The cells were treated with vehicle alone or with the combination of recombinant mouse IL-1β (5 ng/ml), IFN-γ (100 ng/ml) and TNF-α (10 ng/ml) (R&D Systems, Minneapolis, MN, USA) suspended in complete DMEM. A major leucocyte 12LO product, 12(*S*)-HETE and its stereoisomer 12(*R*)-HETE were purchased from Biomol (Plymouth Meeting, PA, USA). Final concentrations of 0–10 μmol/l were achieved by applying their ethanol suspension to the cells. Equal amounts of ethanol were used as vehicle control. The final ethanol concentration in cells was below 0.1%. The incubations of the cells with either cytokines or 12-HETE were in KRB–HEPES buffer containing in mmol/l: 134 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.0 $CaCl_2$, 10 HEPES and 0.1% BSA (fatty acid free; Sigma, St. Louis, MO, USA) at 37°C, pH 7.4 for 0–6 h. A leucocyte 12LO inhibitor, baicalein (5,6,7-trihydroxyflavone, IC_{50} 120 nmol/l; Calbiochem, San Diego, CA, USA), at 0–10 μmol/l was applied to β-TC3 cells and human islets simultaneously with the cytokines.

Static insulin secretion measurement β-TC3 cells were plated in gelatin (2% in sterile saline)-coated plates at a density of $10^5/cm^2$ overnight. Gelatin coating ensured 99.5% of cell attachment even when cell death occurred. At the end of treatment, β-TC3 cells were washed with KRB at 37°C, pH 7.4. They were pre-incubated in the same buffer for 30 min followed by 60 min incubation in

KRB supplemented with 15 mmol/l D-glucose (J. T. Baker, Phillisburg, NJ, USA). The supernatant was harvested and subjected to centrifugation to eliminate cell residue. Insulin secreted into the supernatant was measured using an enzyme immunoassay (EIA) with mouse insulin as a standard.

MTT metabolism in β-TC3 cells Following treatments, β-TC3 cells were washed and pre-incubated in KRB as stated above. The cells were replaced with KRB containing 0.1 mg/ml of MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide; Sigma) with or without 15 mmol/l D-glucose. At the end of 60 min incubation, the supernatants were removed. The insoluble MTT metabolite within the cell monolayer was extracted with isopropanol. The absorbance at 590 nm was recorded using arbitrary optical density units as an indication of the degree of mitochondria metabolism in general [31].

In situ terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling labelling of DNA breakage To assess the cytotoxic effect of 12-HETE on β-TC3 cells, the characteristic endonucleolysis was detected using a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) assay following the manufacturer's instruction (Roche Molecular Biochemicals, Indianapolis, IN, USA). Following treatments, cells grown on culture-grade chamber slides were fed in freshly prepared paraformaldehyde solution in PBS, pH 7.4, for 1 h. Endogenous peroxidase was blocked by incubating the cells in 0.3% hydrogen peroxide in methanol at room temperature. The cells were then permeabilised with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. The TUNEL reaction mixture containing TdT and fluorescein-coupled dUTP was applied to the slides to detect the free 3′ hydroxyl group in broken DNA. The incubation was continued for 1 h at 37°C in a humidified chamber. Horseradish-peroxidase-conjugated anti-fluorescein antibody Fab fragments were used to detect the incorporated fluorescein-dUTP. After substrate reaction, TUNEL-positive cells were observed under a Zeiss light microscope with an RT-Slider CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA). Image-Pro software (MediaCybernetics, Carlsbad, CA, USA) was used to analyse the images and to quantify the percentage of TUNEL-positive cells in each treatment condition.

Immunoblotting Cells were disrupted in RIPA buffer (1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS in phosphate-buffered saline, pH 7.4), supplemented with protease inhibitor cocktail (Sigma-Aldrich) and 1 mmol/l sodium orthovanadate. Protein concentrations in the disrupted cells were determined using a modified BCA assay (Sigma-Aldrich). Samples containing 20 μg of protein were resolved using SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. The membranes were probed with either a rabbit polyclonal antibody raised against purified mouse leucocyte 12LO (kindly provided by Dr Jiali Gu, University of Virginia), or a set of rabbit polyclonal anti-phosphor-specific mitogen-activated kinases (extracellular signal-regulated kinase [ERK]1/2, ERK5, p38 and c-Jun N-terminal kinase [JNK]1/2; Biosource International, Camarillo, CA, USA) (titre 1:2,000) followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection (Amersham Biosciences, Piscataway, NJ, USA). The band intensities, represented as pixel numbers, were measured from the gel images using ImageQuant software (Amersham Biosciences).

Analysis of 12-HETE production 12(*S*)-HETE produced from treated β-TC3 cells was measured using an EIA (Assay Designs, Ann Harbor, MI, USA). Total eicosanoids were extracted from the samples using 0.2 mol/l methanolic sodium hydroxide solution supplemented with 40 mmol/l *n*-propyl gallate (Sigma-Aldrich) to inhibit non-specific NEFA oxidation. Samples were then acidified to pH 3.0–3.5 using 1.0 mol/l hydrochloric acid, followed by C-18 bond elute column extraction (Varian Associates, Harbor City, CA, USA). The purified samples were subjected to an EIA using a specific 12(*S*)-HETE antibody.

12LO subcellular localisation and translocation Cell membrane, cytosol and nuclei subcellular fractions were obtained by subjecting β-TC3 cell homogenates to ultracentrifugation [32]. Cells were treated with the combination of cytokines for 30 min. At the end of the treatment, cell pellets were resuspended in ice-cold PBS (pH 7.4) containing isotonic sucrose (0.25 mmol/l) and 50 μmol/l of EDTA. The cell suspension was then homogenised using a Polytron homogeniser (setting 2) for 30 s on ice. The homogenates were centrifuged at 1,000 *g*, 4°C for 10 min. The pellet was considered the nuclei fraction. The supernatant was then subjected to ultracentrifugation at 40,000 *g* for 30 min in a Beckman 70Ti fixed-angle rotor at 4°C. The pellet was treated as the membrane fraction. The supernatant was designated the cytosolic fraction. As markers for each fraction, DNA content (nucleus) and alkaline phosphodiesterase activity (membrane) were determined and compared with the total homogenate to evaluate the yield and purity of each fraction. The fractions (>tenfold marker enrichment vs total homogenate, purity >50%) were resuspended in RIPA buffer supplemented with protease inhibitors and sodium vanadate. The protein content in the fractions was determined. Ten micrograms of protein per fraction were subjected to immunoblotting using our polyclonal antileucocyte 12LO as the primary antibody. The 12LO band intensity in each fraction was determined using densitometry.

Alternatively, immunofluorescence labelling of cytokine-treated β-TC3 cells with polyclonal anti-12LO was performed to determine the subcellular localisation of 12LO. Cells were grown in tissue-culture-grade chamber slides at the density of $10^4/cm^2$. After treating the cells with the cytokines or vehicle for 30 min, the cells were fixed in methanol at −10°C for 10 min. They were then subjected to immunofluorescence labelling following the standard procedure (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Polyclonal leucocyte 12LO antibody was used

as the primary antibody at the titre of 1:200, and the fluorescein-conjugated anti-rabbit IgG (1:500) was the secondary antibody. The labelled cells were then observed under a Zeiss fluorescence microscope. The images were captured with a CCD camera (RT-Slider; Diagnostic Instruments), and analysed using the ImagePro software (Cybernetics).

Statistics and calculations All numerical data are expressed as means±SEM. The listed *n* values represent the number of experiments performed using β-TC3 cells cultured from different passages. Statistical significance was determined by one-way or two-way ANOVA (depending upon experimental design) coupled with appropriate post-tests to compare replicate means. The null hypothesis was rejected at a *p* value of less than 0.05. The graphic and statistic software Prism (GraphPad Software, San Diego, CA, USA) was used to assist the statistical analysis of all numerical data in this report.

Results

Presence of leucocyte 12LO protein in pancreatic islets and islet cell lines The presence of a leucocyte type 12LO in human and mouse islets has not been evaluated previously. The presence of leucocyte type 12LO is seen in all mouse islet cell lines tested, and in fresh human and mouse pancreatic islets of Langerhans using a relatively specific leucocyte type 12LO antibody (Fig. 1). Blocking experiments were also performed using a partially purified 12LO from mouse leucocytes (Cayman Chemicals Ann Arbor, MI, USA). The 72-M_r band can be effectively blocked off in cell and islet lysates tested (data not shown), suggesting the polyclonal antibody probed a specific band corresponding to the leucocyte type 12LO in human islets and in mouse islet cell lines. Although an equal amount of total protein was present in each lane, the two human islet samples showed visible different levels of 12LO. Since the two human islet samples are from two different donors, and they were stored for different amount of time prior to the experiments, it is difficult to attribute the difference in

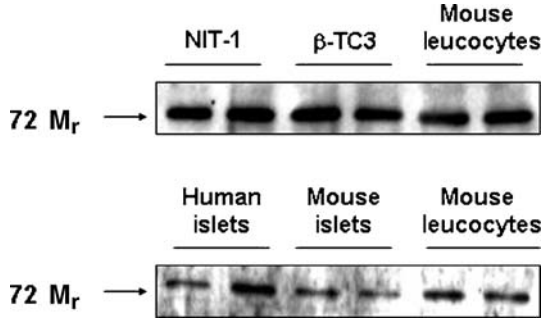

Fig. 1 Presence of leucocyte type 12LO protein in pancreatic islets and islet cell lines. Immunoblotting using a polyclonal anti-leucocyte 12LO revealed the presence of the enzyme in human, mouse islets and in several islet cell lines. *NIT-1* is an insulin-secreting cell line derived from non-obese diabetic mouse pancreas. The gel image shown here is a representation of six independent experiments

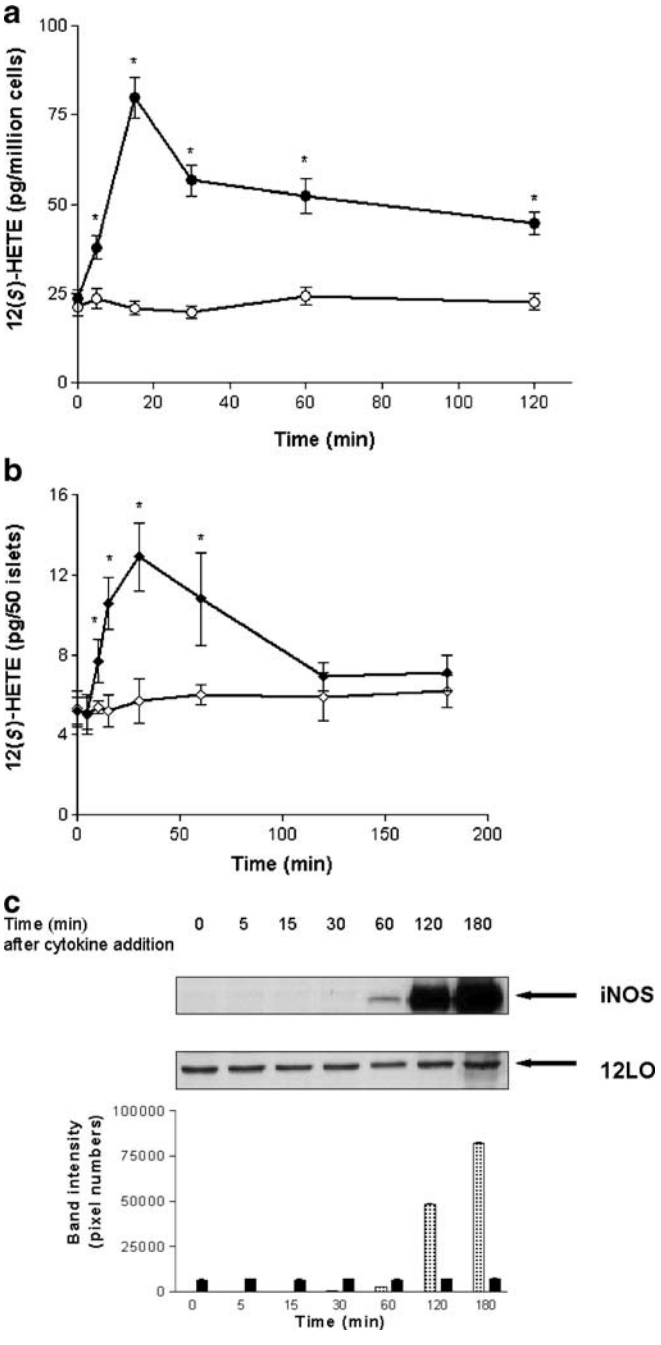

Fig. 2 The effect of cytokines on 12(*S*)-HETE production in β-TC3 cells and human islets. **a** Cells were treated with (*filled circle*) or without (*open circle*) multiple cytokines for 0–20 min. Total eicosanoids were extracted from the cells. 12(*S*)-HETE content was determined using an EIA and was normalised with the cell numbers (means±SEM, *n*=6, **p*<0.01). **b** Fifty human islets per condition treated with the same conditions (*filled diamond*, with cytokines; *open diamond*, without cytokines) for 0–180 min. 12(*S*)-HETE content was determined using an EIA, and was normalised with number of islets (means±SEM, *n*=4, **p*<0.05). **c** β-TC3 cells were also subjected to immunoblotting using a polyclonal anti-leucocyte 12LO antibody and a polyclonal anti-iNOS antibody. The image is representative of four similar blotting experiments. The data in the *bottom graph* (*dotted bar*, iNOS; *filled bar*, 12LO) show the intensity of each band in the four different gels (pixel numbers, means± SEM, *n*=4)

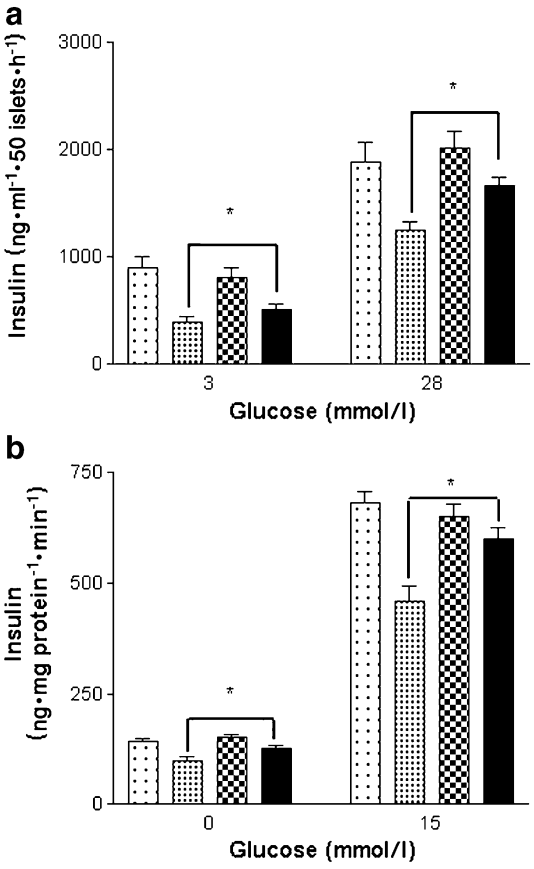

Fig. 3 Effects of baicalein on cytokine-inhibition of insulin release in β-TC3 cells and human islets. Human islets (**a**), and β-TC3 cells (**b**), were treated with species-specific recombinant IL-β, IFN-γ and TNF-α with or without the presence of 0.5 μmol/l of baicalein for 18 h. Static insulin release from the treated samples was measured using an EIA (*sparsely dotted bar*, control; *medium dotted bar*, cytokines; *densely dotted bar*, baicalein alone; and *filled bar*, cytokines and baicalein). n=4, means±SEM, *p<0.05 for cytokine-treated cells compared with baicalein- and cytokine-treated cells in their corresponding glucose concentrations

12LO levels to donor-origin variability or to various degrees of sample degradation.

Cytokine activation of 12LO in β-TC3 cells and human islets The combination of IL-1β, IFN-γ and TNF-α has been shown to inhibit insulin release and cause cell death in islets and cultured beta cells in vitro [33]. IL-1β alone has been shown to induce 12LO expression and activity [18, 20]. To determine whether the combination of cytokines was capable of activating 12LO in β-TC3 cells and human islets, cells were treated with recombinant mouse and human cytokines respectively for 0–3 h in KRB solution with 0.1% BSA and without glucose. The short-term serum-free environment was utilised to reduce the possible influence of other serum factors and cytokines. Intracellular 12(*S*)-HETE production was increased by cytokine treatment in mouse insulin-releasing β-TC3 cells (Fig. 2a), and human islets (Fig. 2b). The liberation of 12(*S*)-HETE by cytokines was rapid (peaking within the first 30 min of treatment), and was sustained above basal level for the entire treatment duration of 2 h. Simultaneous addition of 0.5 μmol/l baicalein blocked 12(*S*)-HETE production (data not shown). Parallel measurement of 12LO protein by immunoblotting, however, indicated that cytokines did not alter cytosolic 12LO protein levels in the same period (Fig. 2c). The increase in 12(*S*)-HETE production and 12LO activation preceded cytokine-induced inhibition of insulin release and cell death, which takes 3–6 h with the same treatment [33]. The results suggest that short-term exposure of cytokines activate 12LO through mechanism(s) independent of the regulation of its protein expression.

Baicalein effects on insulin-release in β-TC3 cells and human islets To determine if 12LO activation contributes to cytokine inhibitory effects on insulin release and cytotoxicity to beta cells and islets, 0.5 μmol/l of baicalein, a 12LO inhibitor, was added simultaneously with the cytokines to β-TC3 cells and dispersed human islet cells for 18 h. Basal and glucose-stimulated insulin release in β-TC3 cells and human islets was determined. Baicalein was able to partially restore insulin secretory function (the restoration rate in β-TC3 cells: basal 27.3%, glucose-stimulated 31.1%; in human islet cells: basal 28.4%; glucose-stimulated 33.3% over cytokine-treated levels, n=4, means ±SEM) (Fig. 3a, b). The result suggests that 12LO activation indeed contributes, at least in part, to the cytokine-induced dysfunction in beta cells.

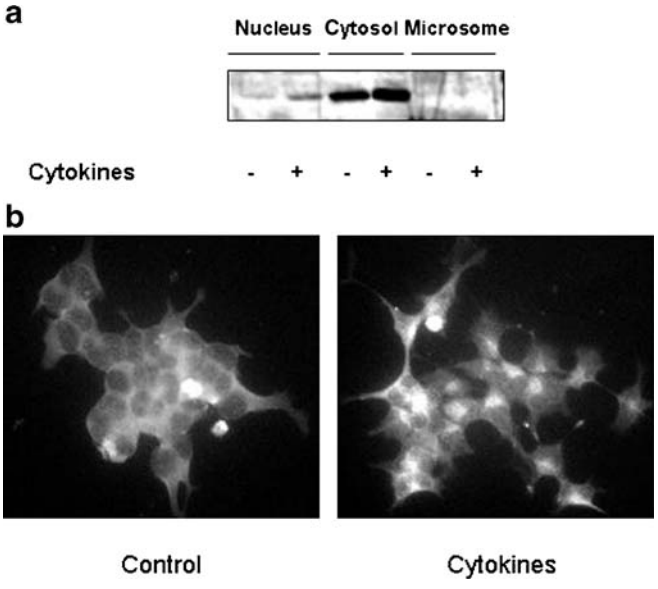

Fig. 4 Cytokine-induced 12LO translocation in β-TC3 cells. **a** Subcellular nucleus, cytosol and microsomal fractions were obtained from cells treated with or without the cytokines. They were subjected to immunoblotting using a polyclonal anti-leucocyte 12LO antibody. The image is representative of four similar experiments. **b** β-TC3 cells treated with cytokines were also subjected to immunofluorescence staining using the same antibody as in immunoblotting. Fluorescein-conjugated anti-rabbit IgG was used as the secondary antibody. The image is representative of six similar staining experiments

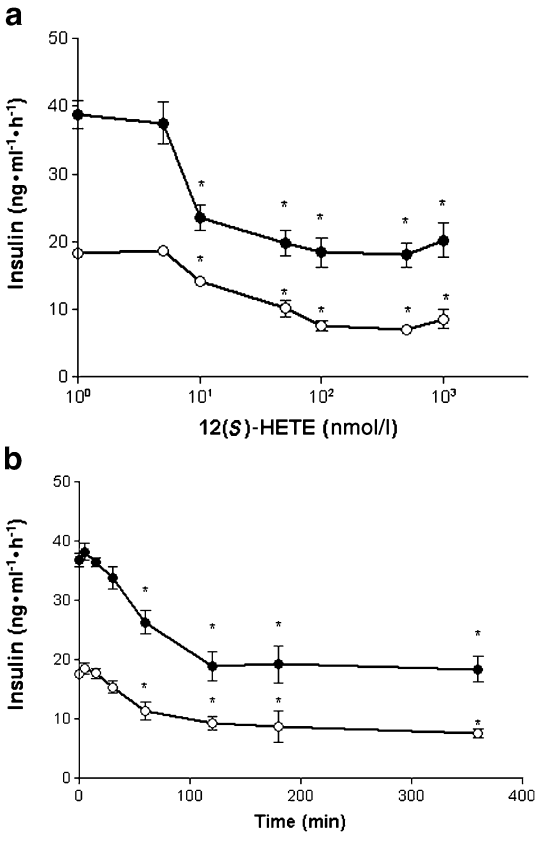

Fig. 5 Effect of 12(*S*)-HETE on static insulin release in β-TC3 cells. Concentration-dependent effect (**a**), and time-dependent effect (**b**), of 12(*S*)-HETE on β-TC3 cells. The cells were incubated with 12(*S*)-HETE (up to 1 μmol/l) for 6 h in KRB solution (*open circle*, no glucose; *filled circle*, 15 mmol/l glucose). The 12(*S*)-HETE incubation time was 2 h (**a**), the concentration of the 12(*S*)-HETE used (**b**) was 100 nmol/l. Static insulin release from the cells was measured using an EIA. n=3, means±SEM, *p<0.01

Cytokines induce 12LO translocation in β-TC3 cells We next explored other possible regulatory mechanisms. One such mechanism is LO translocation to other subcellular compartments. 5LO has been shown to relocate to the nucleus of the leucocytes recruited into the inflammation sites, and 12LO can translocate from the cytosol to plasma membrane in phagocytic macrophages [34, 35]. LO translocation may result in an enhanced activity, and increased lipid hydroperoxide production. Treating β-TC3 cells for 30 min induced leucocyte type 12LO translocation from the cytosol to the nucleus, using subcellular fractionation (Fig. 4a) and immunofluorescence staining of cytokine-treated β-TC3 cells (Fig. 4b). Considering the limitation of subcellular fraction purity, we further examined the 12LO translocation by cytokines using immunofluorescence labelling of the cellular 12LO. The redistribution of fluorescence staining from cytosol to nucleus further supports the notion that cytokines induce the rapid translocation of 12LO (Fig. 4b). In other cells, 12LO translocation to the plasma membrane occurred, but this was not fully apparent in our subcellular fraction analyses.

12(S)-HETE inhibits insulin release in β-TC3 cell To determine the effect of exogenous 12(*S*)-HETE on beta cell function, cells were incubated with 0–10 μmol/l of 12(*S*)-HETE for the duration of 0–6 h in KRB solution. At the end of the incubation, the cells were incubated with 0 or 15 mmol/l glucose for 60 min. Insulin release into the supernatants was measured by the EIA. Concentration- and time-dependent effects of 12-HETE on β-TC3 insulin release cells were determined (Fig. 5a, b). The result shows that 12(*S*)-HETE incubation with the cells inhibited both basal and glucose-stimulated insulin release from β-TC3 cells by 50%. In contrast, 12(*R*)-HETE, a 12(*S*)-HETE isomer not generated by 12LO, did not significantly alter insulin secretion (data not shown).

12(S)-HETE induces β-TC3 cell death Since multiple cytokines can induce β-TC3 cell death and activate 12LO in vitro, we examined the effect of the 12LO product 12(*S*)-HETE on beta cell viability (Fig. 6a, b). The cells were treated with 100 nmol/l of 12(*S*)-HETE for 18 h, in the presence of complete cell growth media. Results from the quantitative in situ TUNEL assay showed that 12(*S*)-HETE-treated cells had a twofold higher cell death rate compared with the control cells (17% vs 5%). The stereoisomer 12(*R*)-HETE, which is not a product of leucocyte 12LO, did not affect β-TC3 viability (data not shown).

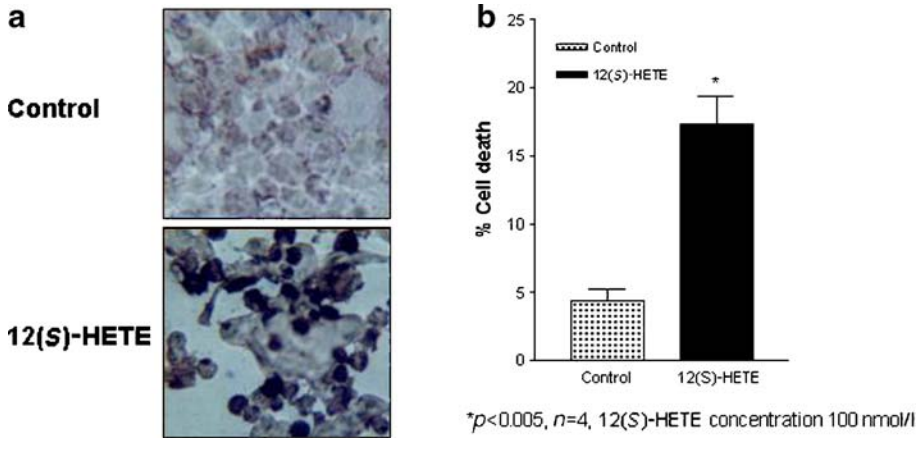

Fig. 6 12(*S*)-HETE-induced β-TC3 cell death. **a** In situ TUNEL labelling of DNA breakage in β-TC3 cells. The image illustrates the dark stained cell nuclei after 18 h of 12(*S*)-HETE treatment. **b** The percentage of cell death observed in 12(*S*)-HETE-treated β-TC3 cells (*dotted bar*, control; *filled bar*, 12-HETE treatment). The number of stained nuclei was counted against the total cell number for five consecutive fields in each experiment condition. n=4, means±SEM, *p<0.005

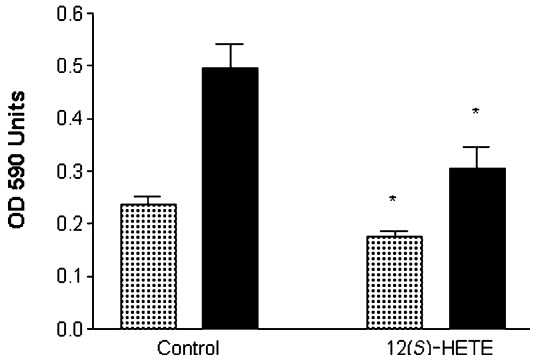

Fig. 7 12(*S*)-HETE inhibited β-TC3 cell mitochondrial metabolism. After treating the cells with 100 nmol/l 12(*S*)-HETE for 6 h, they were incubated in KRB solution containing MTT with or without glucose (*dotted bar*, no glucose; *filled bar*, 15 mmol/l glucose). The MTT metabolites were extracted with isopropanol and measured using a spectrophotometer. n=4, means±SEM, *p<0.01 for 12-HETE-treated cells compared with their corresponding controls in different glucose concentrations

12(S)-HETE inhibits β-TC3 cell mitochondria metabolism
To determine whether exogenous 12(*S*)-HETE has similar effects as proinflammatory cytokines on beta cell energy metabolism, we measured the effect of 12(*S*)-HETE on mitochondrial function in β-TC3 cells as reflected by the cells' ability to reduce MTT under basal and glucose-stimulated conditions. After the supernatant was removed, the MTT metabolite was extracted and analysed. 12(*S*)-HETE inhibited basal and glucose-stimulated MTT metabolism at rates corresponding to its inhibition on insulin secretion (Fig. 7), implicating that the 12(*S*)-HETE inhibition of mitochondrial function may underlie its effect to suppress insulin release.

12(S)-HETE activates mitogen-activated protein kinases
Previous studies indicated that oxidative stress due to diabetes could activate JNK and p38 mitogen-activated protein (MAP) kinase, resulting in reduced insulin-gene expression [36].12LO activation can lead to oxidative stress in target cells. To identify possible signalling mechanisms underlying 12LO cytotoxic effects on beta cells, we measured the level of phosphorylated JNK and p38 as indicators of a kinase activation using phosphor-specific polyclonal antibodies against respective kinases. β-TC3 cells were studied in the presence or absence of cytokines and this was compared with the effect of 12-HETE. The results show that both cytokines and 12(*S*)-HETE were able to induce the phosphorylation of JNK1 (46 M_r) and p38 (Fig. 8a, b). The JNK2 phosphorylation level was not altered in any of the treatment as compared with the control cells. The 12LO inhibitor baicalein decreased the cytokine-induced JNK and p38 phosphorylation, suggesting that the 12LO product 12(*S*)-HETE could be a mediator of cytokine-induced protein kinase activation.

Discussion

12LO expression has been shown to be upregulated in islets and beta cells by the proinflammatory cytokine IL-1β [18], and metabolic stresses [14, 16, 17]. Targeted deletion of leucocyte 12LO also yields protection from low-dose STZ-induced diabetes in the mouse [19]. The previous studies all point to a potentially important role of 12LO in regulating beta cell function and in development of diabetes. The current study provides the first data suggesting that a leucocyte type 12LO is present in human islets and mouse beta cells, and the mechanism of 12LO regulation, especially by proinflammatory cytokines.

Leucocyte type 12LO has been demonstrated to be expressed in mouse and rat islets [8, 9]. In addition, we have preliminary data indicating that insulin-releasing beta cells

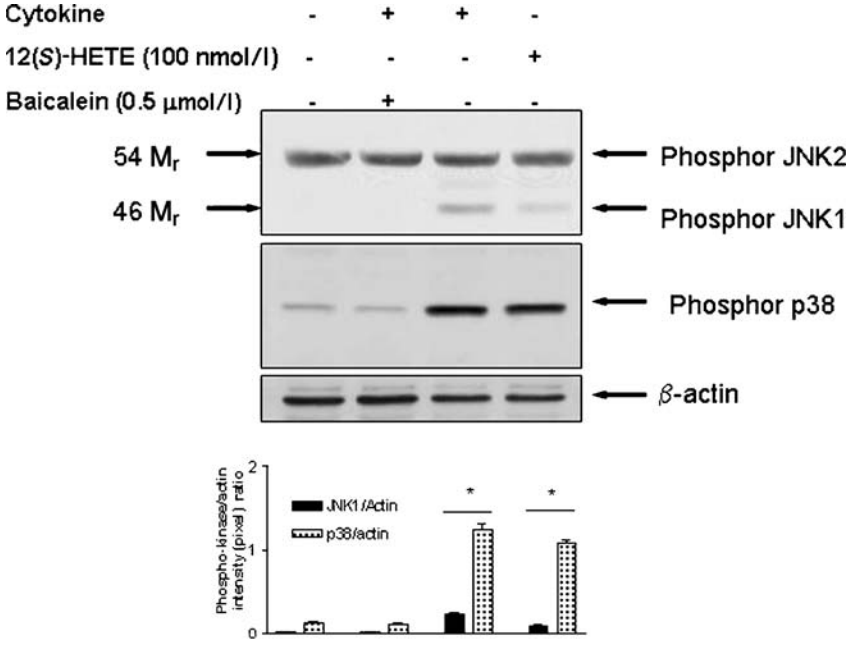

Fig. 8 12(*S*)-HETE and cytokines activated MAP kinase, JNK and p38. β-TC3 cells were treated with 12(*S*)-HETE or multiple cytokines with or without the 12LO inhibitor baicalein for 30 min. The cell protein lysates were subjected to immunoblotting using polyclonal antibodies raised against phosphorylated JNK or p38. β-Actin probe indicates equal sample loading in all lanes. The images represent four independent experiments. The data in the *bottom graph* show the band intensity ratios (JNK1/actin, *filled bar*; or p38/actin, *dotted bar*, *p<0.05 as compared with their corresponding controls where cytokines 12(*S*)-HETE, and baicalein were not present)

express higher levels of 12LO than glucagon-secreting alpha cells (data not shown). It is possible that the presence of leucocyte 12LO particularly in beta cells may contribute to the beta cell intrinsic sensitivity to proinflammatory cytokine damage and oxidative stress [37, 38]. It is also possible that 12LO in beta cells is particularly sensitive to regulation by cytokines.

Several studies have indicated that 12LO expression can be upregulated in pancreatic islets and beta cells. One study showed the increase of 12LO expression by IL-1β in a rat beta cell line [18], while another demonstrated that IL-1 can enhance 12-HETE production in rat islets through release of substrate by activating phospholipase A_2 [20]. Here, we show that short-term incubation of mouse beta cells and human islets with multiple proinflammatory cytokines (IL-1β, IFN-γ and TNF-α) results in a rapid and sustained 12LO activation, while 12LO protein levels remain constant (Fig. 2c). While the data do not exclude the possible long-term regulation of gene expression by cytokines, they indicate an additional immediate activation of 12LO activity by cytokines without the concomitant change in 12LO gene expression. It is likely that 12(*S*)-HETE and other 12LO products are short-lived lipid inflammatory mediators. In addition to producing oxidative stress to beta cells, 12(*S*)-HETE could function as a signalling molecule to trigger and amplify downstream cellular inflammatory processes that are detrimental to beta cell function and viability. The expression of three major isoforms of nitric oxide synthase (NOS) (inducible NOS [iNOS], endothelial NOS and neuronal NOS) has been examined in β-TC3 cells by way of immunoblotting. Only iNOS protein expression was observed in cytokine-treated cells (data not shown). The surge in NO production stimulated by cytokines coincided with the iNOS expression reported in this paper (data not shown). The distinctive protein expression patterns between iNOS and 12LO imply that the cytokines activate 12LO through a post-translational mechanism but not induction of protein expression, at least within the first 180 min of cytokine incubation.

Baicalein, at a concentration of 0.5 μmol/l, inhibits 12LO activity (IC_{50}=120 nmol/l). In the current study, baicalein inhibited the cytokine-induced early phase of 12(*S*)-HETE production in β-TC3 cells and human islets. In addition, baicalein at the same concentration partially restored cytokine-imposed inhibitory effects on β-TC3 cells and human islets. This suggests that 12LO and its lipid products contribute in part to the cytokine-induced inflammatory response in pancreatic beta cells.

Transport of LOs within different subcellular compartments may be one way of modulating enzyme activity. 5LO is imported to the nucleus in leucocytes recruited to the areas of inflammation [35], and 12LO translocation occurs from the cytosol to the plasma membrane of phagocytic macrophages [34]. The current data on 12LO translocation to the nucleus upon cytokine stimulation in β-TC3 cell provide the first demonstration of a novel mechanism of 12LO regulation in beta cells. The crucial nuclear import motif residues are largely conserved among LOs [39], supporting this hypothesis. Relocation of LO to different cellular compartments could result in modulation of LO activity and may be closely related to its biological function in targeting cellular compartments, e.g. modulating nuclear gene transcription activities, or plasma-membrane-related phagocytic activities. The subsequent biological effects of 12LO upon translocation to the beta cell nucleus, as well as the cellular/molecular mechanisms responsible for cytokine-induced 12LO translocation, will be investigated in future studies.

12(*S*)-HETE is a major product of leucocyte type 12LO. Its biological effects have been studied in several cell types [25, 40–42]. The physiological function of low basal levels of 12LO products in the islets remains unclear. Early studies using high concentrations of LO inhibitors suggested that 12LO products play a role in insulin secretion [12]. However, 12LO-null mice exhibited normal release of insulin [19]. In the current study, β-TC3 cells treated with 12(*S*)-HETE for a prolonged period (overnight) showed significantly reduced insulin output. We propose that chronic exposure to proinflammatory cytokines could induce prolonged 12LO activation and 12(*S*)-HETE production, resulting in inhibition of beta cell function. The accompanying mitochondrial inhibition suggests that this subcellular organelle may be a target for 12(*S*)-HETE-imposed cellular oxidative stress and metabolic alterations. 12(*S*)-HETE also induced β-TC3 cell death. Additional studies will be needed to determine whether other independent mechanism(s) are at work in 12(*S*)-HETE-induced beta cell death and functional inhibition. The observed toxic effects of 12(*S*)-HETE to β-TC3 cells were also similar to cytokine-induced damage in pancreatic islets and beta cells. However, multiple cytokines induce a greater rate of cell death than that induced by 12(*S*)-HETE (40% vs 15%). This suggests that cytokines probably have other mechanisms, in addition to activation of 12LO and production of 12(*S*)-HETE, to induce cell death. It is also likely that other inflammatory products of 12LO may be even more potent than 12-HETE in leading to beta cell damage.

MAP kinases are known to participate in cytokine-induced beta cell toxicity [43]. Both JNK and p38 kinases are thought to be critical signalling components in cytokine-induced islet and beta cell death [44–47]. By modulating NOS activity and downstream transcriptional events, the kinases regulate beta cell function and survival. Our results show that 12(*S*)-HETE and cytokines activated both JNK and p38 kinases in β-TC3 cells. In addition, kinase activation by cytokines was blocked by the 12LO inhibitor, baicalein, implicating 12(*S*)-HETE as one mediator of cytokine-induced JNK and p38 kinase activation. The finding that 12(*S*)-HETE activates p38 kinase is consistent with other reports showing that 12(*S*)-HETE treatment or 12LO overexpression induces the p38 phosphorylation in vascular smooth muscle cells [48] and adrenal cells [49]. There are at least ten different isoforms of JNK in mammals. Among them, both JNK1 and JNK2 can independently bind and phosphorylate c-Jun. In cytokine- and/or 12(*S*)-HETE-treated β-TC3 cells, we have observed that the phosphorylation level of JNK2 (54 M_r) remained unchanged,

whereas various treatments changed JNK1 (46 M_r) phosphorylation levels. Our results showing JNK activation by 12-HETE are consistent with a previous study [50]. NO is also known to activate the JNK and p38 kinases. But NO production was delayed as compared with 12(*S*)-HETE production in cytokine-treated β-TC3 cells (data not shown). Since baicalein inhibited JNK and p38 phosphorylation but did not inhibit cytokine-mediated NO production in β-TC3 cells, this suggests that 12(*S*)-HETE production is a specific response to multiple proinflammatory cytokines rather than a general stress response in β-TC3 cells.

In summary, our data provide mechanisms of cytokine regulation of 12LO in pancreatic beta cells, and suggest for the first time the presence of leucocyte 12LO in human islets. We explored the potential of 12(*S*)-HETE, as a signalling molecule, to mediate cytokine-induced protein kinase activation. In the light of recent findings that 12LO deficiency could protect mice from STZ-induced diabetes [19], our study provides mechanistic information supporting the importance of 12LO in beta cell damage during inflammatory attack. Our findings suggest that interventions aimed at blocking 12LO activation and lipid hydroperoxide production could be beneficial in beta cell protection and preservation.

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