ARTICLE

The synthetic liver X receptor agonist GW3965 reduces tissue factor production and inflammatory responses in human islets in vitro

H. Scholz · T. Lund · M. K. Dahle · J. L. Collins ·
O. Korsgren · J. E. Wang · A. Foss

Abstract

Aims/hypothesis Optimising islet culture conditions may be one strategy for reducing islet loss prior to, and immediately after, islet transplantation. Liver X receptor (LXR) agonism has previously been shown to increase insulin release from pancreatic islets and reduce inflammation in leucocytes. Our aim was to investigate whether the synthetic LXR agonist GW3965 could modulate the inflammatory status of human pancreatic islets.

Methods Levels of pro-inflammatory cytokines and tissue factor in isolated human islets were determined by TaqMan low density array and/or real-time quantitative RT-PCR (mRNA levels) and enzyme immunoassay (EIA) (protein levels). Islet viability was measured using intracellular ATP content, ADP/ATP ratio, mitochondrial dehydrogenase activity (XTT assay) and insulin secretion in a dynamic glucose-challenge model. Apoptosis was determined by EIA measurement of histone–DNA complexes present in cytoplasm and by assaying caspase-3/-7 activity.

Results Treatment of LPS-stimulated human islets with the synthetic LXR agonist GW3965 (1 µmol/l) for 24 h reduced mRNA and protein levels of selected pro-inflammatory cytokines (IL-8, monocyte chemotactic protein-1 and tissue factor). Moreover, GW3965 had no adverse effect on insulin secretion, islet viability or apoptosis. No excess of lipid accumulation could be detected with the dosage and exposure time used.

Conclusions/interpretation LXR activation suppresses inflammation in human islets in vitro without adverse effects on islet viability. Short-term moderate activation of LXR prior to islet transplantation may represent a possible strategy for improving post-transplant islet survival.

Abbreviations

ANGPT1	Angiopoietin 1
LPS	Lipopolysaccharide
LXR	Liver X receptor
MCP-1	Monocyte chemotactic protein-1
qRT-PCR	Quantitative RT-PCR
SREBP	Sterol regulatory element-binding protein
TF	Tissue factor
TLDA	TaqMan low density array
VEGF	Vascular endothelial growth factor

H. Scholz (✉) · T. Lund · M. K. Dahle · J. E. Wang · A. Foss
Institute for Surgical Research, Oslo University Hospital,
Sognsvannsveien 20,
0027 Oslo, Norway
e-mail: hanne.scholz@medisin.uio.no

H. Scholz · T. Lund · A. Foss
Division of Surgery, Section of Transplantation,
Oslo University Hospital,
Oslo, Norway

J. L. Collins
GlaxoSmithKline Research and Development,
Research Triangle Park, NC, USA

O. Korsgren
Department of Clinical Immunology, Rudbeck Laboratory,
Uppsala University Hospital,
Uppsala, Sweden

Introduction

Transplantation of pancreatic islets is currently a treatment option for repeated severe hypoglycaemia in type 1 diabetes

mellitus and, as such, the therapy is highly efficient. However, sustained insulin-independence after islet transplantation has been difficult to achieve [1], partly because of a significant loss of islets during brain death, islet isolation, transplantation and revascularisation in the liver [2, 3]. Islet apoptosis may be induced by various stress factors such as hypoxia [4] and inflammation [5]. Optimising islet culture conditions may be one way to reduce islet loss prior to, and immediately after, islet transplantation.

Liver X receptors (LXRα/β), also known as nuclear receptor subfamily 1, group H, member 3 (NR1H3) and nuclear receptor subfamily 1, group H, member 2 (NR1H2), respectively, are ligand-activated transcription factors that belong to the nuclear receptor superfamily, the members of which act as key regulators of lipid metabolism and inflammation (reviewed in [6]). Several studies have suggested a role for LXRs in inflammatory diseases such as atherosclerosis [7], Alzheimer's disease [8] and stroke [9]. In addition, in vivo and in vitro studies have demonstrated that activation of LXR agonists antagonises inflammatory gene expression in mouse macrophages [10], microglia and astrocytes [11], and human monocytes [12]. In cultured murine and human macrophages the synthetic LXR agonist GW3965 has been shown to reduce cytokine-induced tissue factor (TF) production [13] and we have previously shown that GW3965 dose-dependently attenuated LPS-induced release of TNF-α and prostaglandin E_2 by hepatic Kupffer cells in vitro as well as in vivo [14]. Human islets exposed to human blood trigger an instant blood-mediated inflammatory reaction, characterised by platelet consumption and activation of the coagulation and complement systems, and this activation may impair engraftment after intraportal islet transplantation [15].

Tissue factor produced by islets is considered to activate this process and the clinical outcome of islet transplantation is directly related to the extent of TF production [16].

In this early post-transplantation phase, up to 70% of islet mass could be lost in clinical allo-transplantation [17]. Although the pathological and clinical significance of the effects of LXR agonists on insulin-producing cells remains controversial, to date there is no information on the impact of LXR activation on the inflammatory status in human islets. Hence, the aim of this study was to determine the effect of the synthetic LXR agonist GW3965 on TF production and the inflammatory responses in human islets. In the present study, we demonstrate that activation of LXR in vitro decreases *TF* (also known as *F3*) expression and suppresses production of pro-inflammatory cytokines in human islets without adverse effects on islet viability.

Methods

Treatment of cultured islets with GW3965, methylprednisolone (MP) and LPS

GW3965 provided by GlaxoSmithKline, is an synthetic LXR agonist that does not discriminate between LXRα and LXRβ [18], was dissolved in DMSO (0.01%, wt/vol.). Cultured human islets were treated with GW3965, 1 μmol/l, methylprednisolone (SoluMedrol, Pfizer, New York, USA), 1 μg/ml, or the DMSO 'vehicle' only (control) for 24 h, and with lipopolysaccharide (LPS, 1 μg/ml) (serotype 0111:B4, Sigma-Aldrich, Sigma, St Louis, MO, USA) for the last 6 h where indicated.

Islet isolation

Islets were isolated according to the automated method as previously described [19] in the Central Laboratory of the Nordic Network for Clinical Islet Transplantation in

Table 1 Donor and islet preparation characteristics

Donor pancreas	1	2	3	4	5
Age (years)	46	54	55	56	77
Sex	Female	Male	Female	Female	Female
Cold ischaemia time (h)	12.3	3.5	13	6.3	12.5
BMI (kg/m²)	24	29	22	27	21
Pancreas weight (g)	93	133	95	122	90
Yield (IEQ)	83,600	220,000	388,000	335,000	195,000
Purity of fractions used (%)	50 and 65	85 and 90	95	90 and 95	50 and 75
Insulin/DNA content (ng/ml)	7.1	6.9	1.5	5.4	6.1
Insulin stimulation index (SI)	6.1	3.4	9.3	12	6.1
ADP/ATP ratio	0.2	0.19	0.07	<0.05	<0.05
TF content (pmol/μg DNA)	0.025	0.009	0.027	0.022	0.049
MCP-1 (pmol/μg DNA)	0.004	0.011	0.006	0.002	0.012
IL-8 (pmol/μg DNA)	0.013	0.038	0.014	0.008	0.058

IEQ, islet equivalents

Uppsala, Sweden. Donor and islet characteristics are presented in Table 1.

Measurements of insulin secretion by dynamic challenge with glucose

Insulin secretion in response to glucose stimulation was assessed in a dynamic perfusion system. Twenty islets were hand-picked and photographed under a microscope, and initially perfused with 1.67 mmol/l glucose, then with 20 mmol/l, and then again with 1.67 mmol/l. During the 120 min perfusion, fractions were collected at 6 min intervals, immediately ice chilled and stored at −20°C until analysed. Thereafter, the pictures of the different islet preparations were analysed with Cellimage (Friberg, Uppsala, Sweden), total islet size calculated and insulin secretion normalised to pmol l^{-1} insulin 100 islet equivalents^{-1}.

Enzyme immunoassays

Levels of IL-8, monocyte chemotactic protein-1 (MCP-1) (R&D Systems, Minneapolis, MN, USA), TF (Imubind, American Diagnostic, Greenwich, CT, USA), and insulin (Mercodia Insulin ELISA, Uppsala, Sweden) were measured using enzyme immunoassays (EIA) according to manufacturer's instructions. Total protein was measured using standard bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA).

Gene expression analysis

Total RNA was isolated from frozen islet pellets with the RNA Isolation Kit − High Performance and the MagNA Pure LC instrument (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions, and was stored at −80°C. The amount of RNA in all samples was quantitated using a NanoDrop ND-1000 UV/Vis spectrophotometer (Saveen Werner, Malmö, Sweden). Using the High-Capacity cDNA Archive Kit as per the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA), 1 µg total RNA was reverse-transcribed.

TaqMan low density array The use of a TaqMan low density array (TLDA) custom-designed format with a 47+1 (*GAPDH*) configuration (Table 2) from Applied Biosystems was based on our interest in gene expression regulation that, from the literature, appeared to be involved in coagulation, inflammation and apoptosis. A total of 100 µl reaction mixture with 5 µl cDNA template (100 ng mRNA) and 50 µl 2× TaqMan Universal PCR Master Mix (Applied Biosystems) was added to each line of reaction wells after vortex and brief centrifugation. Each reaction well contained 1 µl reaction mixture with 1 ng mRNA. Array cards were sealed with a TaqMan low density array sealer (Applied Biosystems) before centrifugation in a Sorvall Legend centrifuge (Kendro Scientific, Asheville, NC, USA) for 1 min×2 at 12,000 rpm. The PCR amplifications were done in the micro-fluidic card sample block of an ABI Prism 7900HT sequence detection system (Applied Biosystems). The amplification protocol was: 2 min at 50°C to activate uracil–DNA glycosylase, 10 min at 94.5°C (activation), 40 cycles of denaturation at 97°C for 30 s, and annealing and extension at 59.7°C for 1 min. All experiments were analysed on array cards with the same batch number. The relative amount of each target gene mRNA to *GAPDH* was calculated as difference in threshold cycle number (C_t), $\Delta C_t = C_t$ Target $- C_t$ *GAPDH*. The threshold cycle C_t was automatically given by the SDS2.2 software package (Applied Biosystems) and C_t values above 35 were considered absent. The mRNA expression levels of GW39865-exposed islets and control islets were displayed as three groups; high ($\Delta C_t < 2$), medium (ΔC_t 2–8) and low ($\Delta C_t > 8$). For the calculation of the differential expression in GW3965-exposed islets compared with controls, the ΔC_t value for GW3965-exposed islets was divided by ΔC_t-value for controls. Trends obtained were used for targeted real-time quantitative RT-PCR (qRT-PCR).

Real-time quantitative RT-PCR

Real-time qRT-PCR using sequence-specific primers was performed on an ABI Prism 7900HT sequence detector (Applied Biosystems) using 2× qPCR Master Mix for SYBR Green (Eurogentec, Liege, Belgium), 300 nmol/l sense and antisense primers, 2.5 µl cDNA template (100 ng mRNA), and water to bring the volume to 25 µl. The specificities of all SYBR Green assays were confirmed by melting-point analysis. Levels of *GAPDH* mRNA were used for normalisation of target-gene expression. All samples were run in duplicate using the following primers: *GAPDH*: forward 5′-CCAAGGTCATCCATGACAACTT-3′, reverse 5′-AGGGGCCATCCACAGTCTT-3′; *LXR-α* (also known as *NR1H3*): forward 5′-TCAGCATCTTCTCTGCA GACCGG-3′, reverse 5′-TCATTAGCATCCGTGGGAACA; *LXR-β* (also known as *NR1H2*): forward 5′-CTGCTAGAGA CAGCCAGGC, reverse 5′-AAGATGTTGATGGCGATGAG; *ABCA1*: forward 5′-GGTGTCAAACAGCATCATGG, reverse 5′-CTCCAGGGGAAATGTCAGAA; *ABCG1*: forward 5′-GACTCGGTCCTGACACACCT, reverse 5′-CTCCAGGGGAAATGTCAGAA; *SREBP-1c* (also known as *SREBF1*): forward 5′-GGAGGGGTAGGGCCAAC, reverse 5′-GTCAAATAGGCCAGGGAAGTC; *TF*: forward 5′-GCGCTTCAGGCACTACAAAT-3′, reverse 5′-

Table 2 Effect of LXR agonist on islet gene expression and regulation in a TaqMan low density array

Gene	Assay ID	Mean expression level	ΔC_t GW3965/ΔC_t control		
			Mean	SD	p value
Genes that tended to be downregulated by GW3965					
IL8	Hs00174103_m1	High	1.30	0.36	0.05[*]
GCG	Hs00174967_m1	High	1.01	0.04	0.75
STAT1	Hs00234829_m1	Medium	1.16	0.15	0.02[*]
NFKB1	Hs00765730_m1	Medium	1.00	0.07	0.90
ICAM1	Hs00164932_m1	Medium	1.24	0.09	0.06
MCP-1	Hs00234140_m1	Medium	1.18	0.13	0.01[**]
SMAD3	Hs00232222_m1	Medium	1.03	0.08	0.32
EGFR	Hs00193306_m1	Medium	1.01	0.05	0.75
IL1B	Hs00174097_m1	Medium	1.01	0.04	0.61
TGFB1	Hs99999918_m1	Medium	1.01	0.67	0.66
CASP8	Hs00154256_m1	Low	1.02	0.04	0.21
TNF	Hs00174128_m1	Low	1.00	0.03	0.69
CCL3	Hs00234142_m1	Low	1.01	0.06	0.76
CCL5	Hs00174575_m1	Low	1.17	0.14	0.01[**]
JAK1	Hs00233820_m1	Low	1.05	0.12	0.29
SLC30A10	Hs00218883_m1	Low	1.01	0.05	0.62
C5	Hs00156197_m1	Low	1.00	0.04	0.84
IL6	Hs00174131_m1	Low	1.22	0.37	0.13
BCL2	Hs00153350_m1	Low	1.08	0.12	0.10
IL10	Hs00174086_m1	Low	1.00	0.10	0.57
Genes that tended to be upregulated by GW3965					
INS	Hs02741908_m1	High	0.98	0.05	0.78
TLR4	Hs00152939_m1	Medium	0.86	0.11	0.03[*]
DIABLO	Hs00219876_m1	Medium	0.99	0.08	0.71
CD40	Hs00386848_m1	Medium	0.99	0.08	0.71
SLC39A1	Hs00205358_m1	Medium	0.69	0.13	0.002[**]
BAX	Hs00180269_m1	Medium	0.99	0.12	0.81
ACTB	Hs99999903_m1	Medium	0.49	0.19	0.001[**]
INSR	Hs00169631_m1	Medium	0.94	0.09	0.10
VEGFA	Hs00900054_m1	Medium	0.91	0.11	0.05[*]
TXNIP	Hs00197750_m1	Medium	0.94	0.25	0.53
MAPK1	Hs00177066_m1	Medium	0.81	0.32	0.14
CCL4	Hs99999148_m1	Medium	0.98	0.27	0.87
CXCL10	Hs00171042_m1	Low	0.78	0.25	0.05[*]
BAD	Hs00188930_m1	Low	0.95	0.07	0.06
IL15	Hs00542562_m1	Low	0.91	0.12	0.07
FOXP3	Hs00203958_m1	Low	0.91	0.12	0.07
CCR5	Hs00152917_m1	Low	0.95	0.09	0.38
SSTR5	Hs00265647_s1	Low	0.98	0.12	0.66
ANGPT1	Hs00181613_m1	Low	0.77	0.12	0.001[**]
CASP3	Hs00234385_m1	Low	0.96	0.05	0.07
CXCL12	Hs00930455_m1	Low	0.99	0.07	0.63
IL18	Hs00155517_m1	Low	0.88	0.24	0.20
FAS	Hs00163653_m1	Low	0.97	0.20	0.70
CASP9	Hs00154261_m1	Low	0.89	0.13	0.06

Table 2 (continued)

Gene	Assay ID	Mean expression level	ΔC_t GW3965/ΔC_t control		
			Mean	SD	p value
Genes that were not expressed					
CCR2	Hs00174150_m1	–	–	–	–
IL4	Hs00174122_m1	–	–	–	–
IFNG	Hs00174143_m1	–	–	–	–

Data are mean±SD

Expression levels are based on the individual ΔC_t values, denoted as high, medium and low as described in the methods section. No expression is shown as –

Six different fractions from three independent donors were tested, with purity ranging from 90% to 95%

Significant differences compared with the vehicle group: [*] $p<0.05$, [**] $p<0.01$

TTTGCTTTTCCAATCTCCTGA-3′; *IL8*: forward 5′-GCCAACACAGAAATTATTGTAAAGCTT-3′, reverse 5′-CCTCTGCACCCAGTTTTCCTT-3′; and *MCP-1* (also known as *CCL2*): forward 5′-AAGCTGTGATCTTCAA GACCATTGT-3′, reverse 5′-TGGAATCCTGAACC CACTTCTG-3′. Standard curves were run on the same plate, and the standard curve method was used to calculate the relative gene expression.

Lipid staining (Oil-Red-O) and lipid-mass quantification

One hundred hand-picked islets were incubated with or without test compounds for 24 h. After incubation, islets were washed once in ice-cold PBS, which was followed by formaldehyde fixation (2% in PBS) for 30 min at room temperature. Neutral lipids were stained using 0.5% Oil-Red-O (Sigma) in isopropanol for 60 min. The Oil-Red-O-stained lipids were measured spectrophotometrically after isopropanol extraction, or morphologically evaluated by microscopy. In parallel experiments cellular content of total cholesterol and triacylglycerols was measured colorimetrically by an enzymatic assay (BioMérieux, Marcy-l'Etoile, France) after hexane–isopropanol extraction of 100 hand-picked islets.

Assessment of islet viability and purity

Mitochondrial function Cell viability was assessed using a combination of the ATP luminescence-based assay CellTiter-Glo (Promega, Mannheim, Germany) for ATP content, and the colorimetric XTT tetrazolium assay (Roche Diagnostics, Mannheim, Germany) for total dehydrogenase activity. The ApoGlow kit (Cambrex Bio Science, Nottingham, UK) was used for ADP/ATP determination according to the manufacturer's instructions as previously described [20]. One hundred hand-picked islets were cultured with or without test compounds for 24 h. Samples prepared as per kit instructions were plated in an opaque-walled plate and placed on an

orbital shaker for 2 min, then equilibrated at room temperature for 10 min and read using a TECAN GENios plate reader with the Magellan version 6.0 software (Tecan Group, Männedorf, Switzerland). Serially diluted ATP (Sigma-Aldrich) was used as a standard in both the CellTiter-Glo and the ApoGlow assays. In parallel experiments, islets were co-cultured during the last 6 h with 75 μl of XTT-labelling reagent (Boehringer Mannheim, Mannheim, Germany; final concentration 0.3 mg/ml). This assay is based on the conversion of the tetrazolium salt XTT into an orange-red coloured formazan product by mitochondrial enzymes. The optical densities were monitored spectrophotometrically at 492 nm using the TECAN GENios plate reader.

Apoptosis measurement The apoptotic response was measured using Apo-ONE Caspase-3/-7 assay (Promega, Mannheim, Germany) and the Cell Death Detection ELISA[PLUS] (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Briefly, 100 hand-picked islets were incubated for 24 h with or without test compounds. After incubation, islets were washed and transferred into a 96-well plate (20 islets/well) for caspase-3/-7 activity, measured as an increase in the rhodamine 110 fluorescence (excitation 499 nm and emission 521 nm) after cleavage of the caspase substrate Z-DEVD-rhodamine, or lysed at room temperature for 30 min and then transferred into a 96-well plate (20 μl), for apoptotic cell death measurement by the detection of DNA–histone complexes present in the cytoplasmic fraction of cells. Absorbance of samples was read spectrophotometrically at 405 nm using the TECAN GENios plate reader.

Statistical analysis

The data in Table 2 are expressed as mean±SD, whereas all other data are expressed as mean±SEM. Statistical signif-

icance was determined using a one-way ANOVA with a post hoc Bonferroni's test or Student's t test. Differences were considered significant when $p<0.05$.

Results

LXRs are produced in human pancreatic islets: effect of LPS, GW3965 and methylprednisolone on expression levels

Real-time qRT-PCR analysis showed that LXR-α and -β were produced in human islets at similar levels, and the production of LXRs in islets was compared with that in human monocytes, where LXR production is documented [12] (Fig. 1a, b). Additionally, exposure to LPS (1 μg/ml, duration 6 h) resulted in a significant increase in production of both LXR-α and -β in human islets compared with unexposed controls (1.6- and 1.7-fold increase, respectively, $p<0.05$) (Fig. 1a, b). Interestingly, addition of the LXR agonist GW3965 (1 μmol/l) 18 h prior to LPS exposure resulted in an additional increase in the expression of $LXR\text{-}\alpha$ and $LXR\text{-}\beta$ (2.0- and 2.3-fold increase compared with LPS-

exposed controls, respectively, $p<0.05$; Fig. 1a, b). Because LXRs have a profound effect on serum glucocorticoid levels and secretion from the adrenal gland [21], we evaluated the effect of glucocorticoids on the expression levels of $LXR\text{-}\alpha$ and $LXR\text{-}\beta$. However, we found no evidence for an effect of the glucocorticoid methylprednisolone, 1 μg/ml, on the expression of these genes in human islets (Fig. 1a, b). These data indicated that LXRs are produced in human islets, and that LPS increases the level of production. Additionally, the data indicate that the LXR agonist GW3965 further upregulated LXR production when measured after 24 h. Glucocorticoids (methylprednisolone), however, did not have an effect on $LXR\text{-}\alpha$ and $LXR\text{-}\beta$ expression, suggesting that the immunosuppressive effects of glucocorticoids are independent of LXR activation.

Effects of GW3965 on human islet lipid homeostasis

Next, we evaluated the effect of GW3965 on well-known LXR target genes by real time qRT-PCR. As expected, the LXR agonist GW3965 (1 μmol/l) potently induced the expression of the genes encoding cholesterol efflux trans-
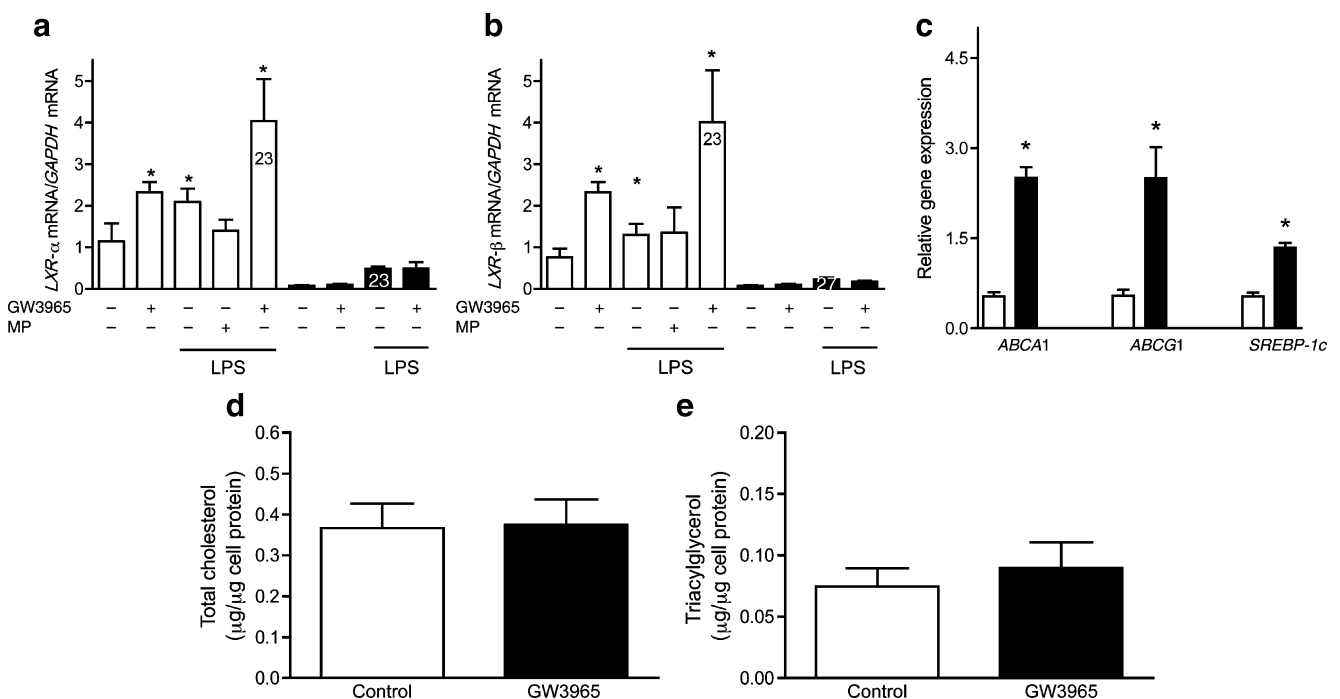
Fig. 1 Effect of LXR agonist on cholesterol homeostasis in human pancreatic islets. Levels of $LXR\text{-}\alpha$ (**a**) and $LXR\text{-}\beta$ (**b**) mRNA from human islets (white bars) treated without or with LXR agonist (GW3965), 1 μmol/l, or methylprednisolone, 1 μg/ml, for 24 h, and as a positive control from human monocytes (black bars) treated without or with LXR agonist (GW3965), 1 μmol/l, for 3 h were analysed using real-time RT-PCR assays and normalised to $GAPDH$ mRNA levels. Where indicated, 1 μg/ml LPS was added to the pancreatic islets at $t=18$ h and to the monocytes at $t=1$ h. Cycle times for the highest-expressing group for each gene are shown in their corresponding bar. **c** Human islets were treated without (control, white bars) or with 1 μmol/l LXR agonist (GW3965, black bars) for 24 h. RNA was prepared and subjected to real-time qRT-PCR analysis of LXR target genes $ABCA1$, $ABCG1$ and $SREBP\text{-}1c$. The expression levels were normalised to $GAPDH$ levels. Cholesterol (**d**) and triacylglycerol (**e**) content normalised to total protein content in human islets after LXR agonist treatment (GW3965), 1 μmol/l, or vehicle for 24 h using EIA as described in the methods section. $n=10$ different fractions from five independent donors, with purity ranging from 50% to 95%. $^*p<0.05$, significantly different from vehicle-treated samples. Data represent the mean±SEM. MP, methylprednisolone

porters ATP-binding cassettes A1 and G1 (ABCA1 and ABCG1) ($p<0.05$) and the master regulator of fatty acid metabolism, sterol regulatory element-binding protein (SREBP-1c), ($p<0.05$) in human islets (Fig. 1c). It has been shown that the LXR agonist T0901317 induces severe lipogenesis [22] and that LXR-deficient mice exhibit reduced NEFA levels and improved metabolic control [23]. We therefore examined whether the LXR agonist GW3965 had effects on lipid homeostasis on human islets. We found no evidence of excess cholesterol (Fig. 1d) or triacylglycerol (Fig. 1e) stored in human islets at the concentration used in our experiments. In addition, Oil-Red-O staining showed no difference in the accumulation of neutral lipids in GW3965-exposed islets compared with control islets (data not shown).

Effect of GW3965 on human islet gene expression in TLDA

We analysed the expression of genes encoding anti- and pro-inflammatory markers, as well as markers associated with islet viability and insulin synthesis, using a TLDA (Table 2) after 24 h of GW3965 (1 μmol/l) or vehicle exposure. LPS (1 μg/ml) was added at $t=18$ h. As shown in Table 2, GW3965 significantly downregulated the expres-

sion of genes encoding several pro-inflammatory mediators and cytokines (MCP-1, chemokine [C-C motif] ligand 5 [CCL5] and IL-8) as well as the transcription factor signal transducer and activator of transcription 1 (STAT1). On the other hand, GW3965 upregulated expression of the gene encoding toll-like receptor 4 (TLR4), a receptor mediating the recognition of LPS and an activator of the innate immune system [5]. Expression of the gene encoding pro-inflammatory cytokine chemokine (C-X-C motif) ligand 10 (CXCL10) was also upregulated, this cytokine having previously been shown to play a role in infection of human islets [24]; however, the expression level was very low and needs to be verified. Of note, the pro-angiogenetic factor angiopoietin 1 (ANGPT1) and vascular endothelial growth factor (VEGF) were upregulated as well as the zinc transporter solute carrier family 39, member 1 (SLC39A1), reported to be produced in MIN6 cells and mouse islets [25]. These data suggest that LXR activation using GW3965 affects the inflammatory profile of human islets.

Effect of GW3965 on key inflammation markers in human islets

Because cytokine-induced islet dysfunction probably plays an important role in islet survival after transplantation [5],
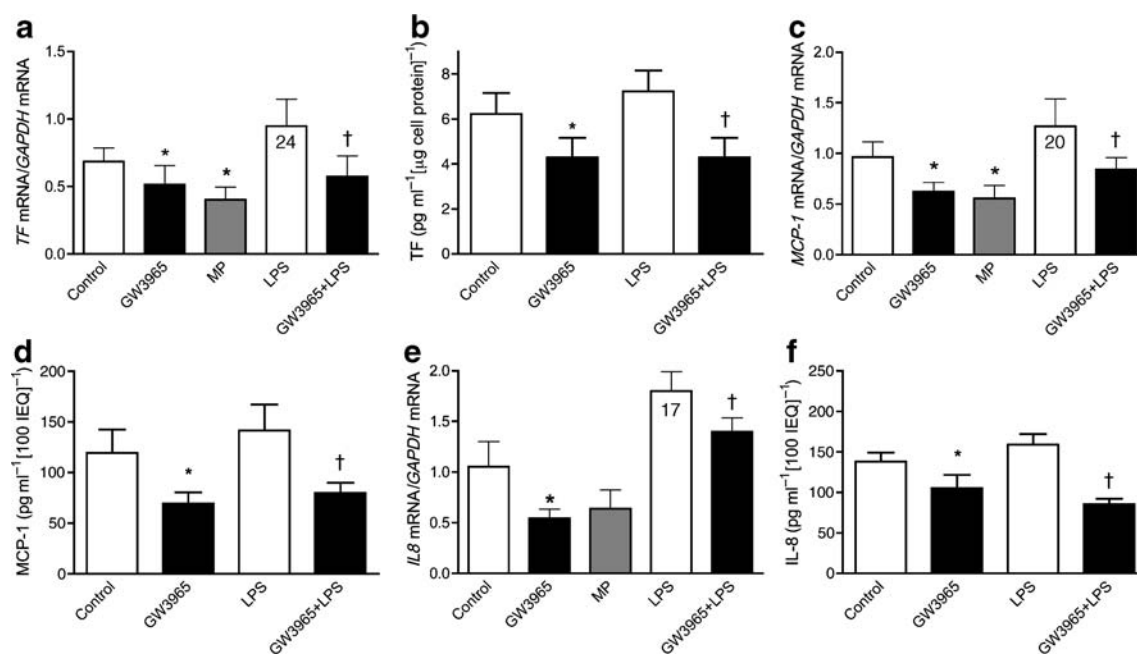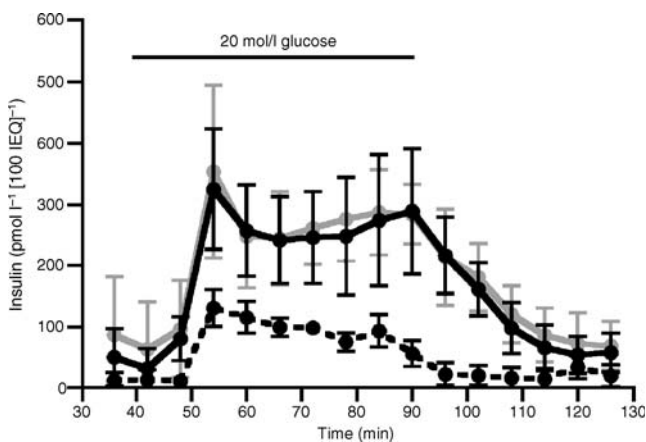

Fig. 2 Effect of LXR agonist on key inflammatory mediators in human pancreatic islets. Islets were treated without or with LXR agonist (GW3965, black bars), 1 μmol/l, or methylprednisolone (grey bars), 1 μg/ml, for 24 h. Where indicated, 1 μg/ml LPS was added at $t=18$ h. Levels of mRNA were analysed using real-time qRT-PCR and normalised to *GAPDH* mRNA levels. Cycle times for the highest-expressing group for each gene are shown in their corresponding bar. Protein levels were analysed using EIA as described in the methods.

Results for the control, vehicle-treated islets, are shown as white bars. *TF* mRNA (**a**) and intracellular of TF protein (**b**). *MCP-1* mRNA (**c**) and MCP-1 protein in the supernatant fraction (**d**). *IL8* mRNA (**e**) and IL-8 protein in the supernatant fraction (**f**). $n=10$ islet preparations from five independent donors, with purity ranging from 50% to 95%. Data represent the mean±SEM. *$p<0.05$, significantly different from control; †$p<0.05$, significantly different from LPS-stimulated control. IEQ, islet equivalents; MP, methylprednisolone
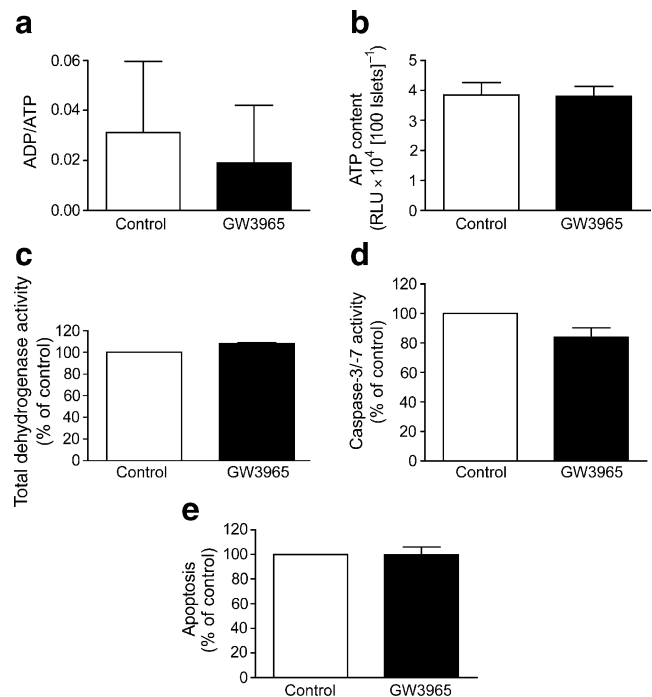
we chose to further evaluate anti-inflammatory effects of the LXR agonist GW3965 in human islets. Levels of TF and MCP-1 have been shown to correlate with islet post-transplant function [26], and *IL8* was the highest expressed gene encoding a pro-inflammatory cytokine in our TLDA (Table 2). Therefore, these three mediators were chosen for further evaluation. Methylprednisolone has been shown to have potent anti-inflammatory properties in human islets [27] and was therefore used as a positive control. As depicted in Fig. 2a–f, islets exposed to GW3965 (1 μmol/l) for 24 h showed a significantly reduced mRNA expression of *TF* (Fig. 2a), *MCP-1* (Fig. 2c) and *IL8* (Fig. 2e) (1.3-, 1.5- and 1.9-fold reduction, respectively, $p<0.05$) compared with controls, analysed by real-time qRT-PCR. Similarly, when GW3965-exposed islets were stimulated with LPS (1 μg/ml) at $t=18$ h, we found significantly reduced mRNA expression of *TF* (Fig. 2a), *MCP-1* (Fig. 2c) and *IL8* (Fig. 2e) (1.5-, 2.2- and 2.5-fold reduction, respectively, $p<0.05$) compared with LPS-stimulated controls. These gene-expression data were confirmed with protein levels, either by measurement of intracellular TF (Fig. 2b) or levels of MCP-1 (Fig. 2d) and IL-8 (Fig. 2f) in the supernatant fractions. As expected, treatment of islets with methylprednisolone for 24 h significantly reduced mRNA expression of *TF* (Fig. 2a) and *MCP-1* (Fig. 2c), but not *IL8* (Fig. 2e).

Effect of GW3965 on insulin secretion from human islets

Several immunomodulatory drugs [27–29] have negative effects on insulin secretion from human islets; however, the

Fig. 4 Effect of LXR agonist on human islet viability. Islets were treated with LXR agonist (GW3965, black bars), 1 μmol/l, or vehicle (control, white bars) for 24 h and subjected to analysis as described in the method section. **a** ADP/ATP ratio measured using the ApoGlow kit. **b** ATP content in islet lysates measured by a CellTiter-Glo bioassay. **c** Total dehydrogenase activity (XTT assay). **d** Caspase-3/-7 activity measured by the Apo-ONE caspase assay. **e** Apoptotic cell death measured by Cell Death Detection ELISA^{PLUS}. Data are calculated as percentages of control and are presented as mean± SEM. $n=10$ fractions from five independent donors, with purity ranging from 50% to 95%. RLU, relative luminescence units

Fig. 3 Effect of LXR agonist on insulin secretion from human pancreatic islets. Islets were treated with LXR agonist (GW3965), 1 μmol/l, (black line), methylprednisolone, 1 μg/ml, (dotted line) or vehicle (grey line) for 24 h. Twenty islets were hand picked, photographed and subjected to dynamic glucose challenge as described in the methods section. Insulin was measured with EIA and normalised to islet area using Cellimage. $n=6$ islets picked from pure fractions (90–95% purity) originating from three independent donors. Data represent the mean±SEM. IEQ, islet equivalents

effect of GW3965 has not been tested. We therefore treated human islets with GW3965, methylprednisolone or vehicle for 24 h and tested the insulin secretory response in a dynamic assay (Fig. 3). No difference in the shape, maximal insulin peak or AUC insulin could be detected between islets treated with GW3965 (1 μmol/l) or with vehicle (Fig. 3 and data not shown). However, when calculating insulin stimulation index (defined as the ratio of secreted insulin at 1.67 mmol/l glucose to secreted insulin at 20.0 mmol/l glucose), the index for GW3965-treated islets was slighter higher than that for control islets (5.6 vs 4.5, $p<0.05$), reflecting a tendency towards lower basal insulin secretion. As expected, islets treated with GW3965 and control islets had larger maximal insulin peaks, AUC insulin values and insulin stimulation indices compared with islets treated with methylprednisolone (1 μg/ml) (Fig. 3 and data not shown).

These data indicate that the LXR agonist GW3965 does not have negative effects on insulin secretion from human islets.

Effects of GW3965 on human islet viability

As high dosages of LXR agonists have been shown to adversely affect murine islet viability [30], we evaluated the effect of LXR activation on human islet viability using different methods. As shown in Fig. 4a–e, in human islets none of the following was adversely affected by GW3965 (1 μmol/l) treatment for 24 h: (1) ADP:ATP ratio; (2) ATP content; (3) total dehydrogenase activity; (4) caspase-3 and -7 activity; (5) or apoptosis. Taken together, these data indicate that the concentration of GW3965 used in the present study does not affect human islet viability.

Discussion

Acute and chronic loss of islet function due to inflammatory processes (reviewed in [31]) is one of the main problems in clinical islet transplantation. Islets are usually cultured for 24–48 h prior to transplantation, and during this time period, modification of the islet inflammatory profile represents a possible strategy for improving islet function post-transplant [32, 33]. Liver X receptors are activated by endogenously produced oxysterols, and synthetic compounds such as T0901317 and GW3965 [34] have emerged as important regulators of inflammation [35]. We chose the selective LXR agonist GW3965 to explore its effects on the inflammatory status of human islets in vitro.

First, we showed that both LXR-α and -β are produced in human islets. This is in agreement with previous studies showing that human and rodent islets produce both the LXR-α and LXR-ß isoforms [36]. We found that the production of LXRs was upregulated by LPS stimulation (Fig. 1a, b), suggesting that activation of LXRs during stress constitutes an endogenous protective mechanism in human islets, as has been found to be the case in macrophages [13] and human monocytes [12]. Adding GW3965 to LPS-stimulated human islets further upregulated the levels of LXRs (Fig. 1a, b). In agreement with our findings, the study by Meng et al. also showed a significant upregulation of LXRs by the addition of LXR agonists to mouse beta cells [37]. Furthermore, we showed that GW3965 significantly induced the expression of the typical LXR lipogenic target gene, SREBP-1c, as well as the cholesterol efflux genes ABCA1 and ABCG1 in LPS-stimulated islets (Fig. 1c). As prolonged activation of lipogenic gene expression can result in accumulation of NEFA and triacylglycerols, causing beta cell dysfunction [38], we measured the total lipid accumulation as well as intracellular triacylglycerols and cholesterols in GW3965-exposed islets. In our study, however, we found no evidence of storage of excess lipids in GW3965-exposed islets (Fig. 1d, e), and consequently no adverse effects on

islet viability (Fig. 4a–e). It has been established that LXR is important for maintaining normal beta cell function [39], but compelling evidence has shown that abnormal activation of LXR could also induce islet lipotoxicity [30, 40]. A possible explanation may be the LXR agonist used (GW3965 in our study, T0901317 in the studies by Wente et al. [40] and Choe et al. [30]), as T0901317 has also been shown to be a potent pregnane X receptor ligand [41]. A second explanation may be the concentrations and/or exposure time used in the experiments, as we used both a relatively modest concentration and a relatively short incubation time (24 h). A third explanation for the diverging results may be the glucose concentrations used in the experiments (5 mmol/l glucose in our experiment, 11.2 mmol/l in the experiment conducted by Choe and co-workers). Consequently, Choe and co-workers enhanced the adverse LXR effects when increasing the glucose concentrations to 31.2 mmol/l in their work [30]. Finally, we cannot rule out species-specific differences in the effects of LXRs, as murine islets or murine insulinoma cells were used by Choe et al. [30] and Wente et al. [40] whereas human pancreatic islets were used in our experiment. Such species differences may apply to pancreatic islets [27, 37].

Pro-inflammatory cytokines produced by cells as part of the innate immune response that infiltrate transplanted islets have important implications for islet survival and function after transplantation [42]. No studies to date have investigated the effects of LXR activation in human islets with focus on the inflammatory status. Using TLDA we obtained gene expression profiles for 47+1 (GAPDH) anti- and pro-inflammatory markers, as well as markers associated with islet viability and insulin synthesis, to map the short-term effect of GW3965 on LPS-stimulated human islets. We observed a significant decrease of the mRNA expression of the pro-inflammatory mediators IL-8, MCP-1 and chemokine (C-C motif) ligand 5 (RANTES) (Table 2). Moreover, because TF is also thought to play an important role in the clinical outcome after islet transplantation [43], we included TF in addition to MCP-1 and IL-8 in the subsequent analysis of pro-inflammatory mediators in human islets in response to GW3965 under normal conditions and after LPS stimulation using real-time qRT-PCR and EIA as detailed in the methods section. We found that mRNA expression of TF, MCP-1 and IL8 was significantly reduced, as were the amounts of the proteins produced in response to LXR activation (Fig. 2a–f). These data suggest that the local inflammatory response in human islets is lowered by short-term LXR activation.

Interestingly, the TLDA data also showed significant upregulation of two known pro-angiogenic factors ANGPT1 and VEGF in GW3965-exposed islets (Table 2). These factors have been shown to promote graft angiogenesis and enhance islet revascularisation, contributing to

improved glycaemic control and better preservation of islet mass in mice [44, 45]. Moreover, we also found that LXR activation significantly decreased the transcription factor STAT-1, known to be involved in cytokine-induced beta cell death [46]. On the other hand we observed upregulation of TLR4, a receptor associated with enhanced bacterial removal during infection. These data suggest that the endogenous effects of LXRs also apply to human islets, on one hand limiting excessive cytokine production and on the other hand enhancing removal of gram-negative bacteria during infection (reviewed in [47]). However, TLR4 has also been implicated in beta cell death and graft rejection after transplantation [48]. The role of these mediators in the recruitment of inflammatory cells and angiogenesis in islets warrants further studies.

To evaluate islet viability, we measured insulin secretion from islets exposed to GW3965 and vehicle in a dynamic model. No difference in net insulin secretion or maximal insulin peaks could be detected (Fig. 3). However, there was a tendency towards lower basal insulin secretion, resulting in an increased insulin stimulation index. Increased basal insulin secretion is associated with cytokine-induced islet dysfunction [49], and these data may imply that GW3965-induced reduction in inflammatory milieu has positive effects on human islets in vitro. We also evaluated the effect of GW3965 on mitochondrial dehydrogenase and caspase-3 and -7 activities as well as apoptosis, and found no adverse effects of GW3965 (Fig. 4c–e).

Taken together, the data obtained in this study show that short-term (24 h) activation of LXRs with the synthetic LXR agonist GW3965 has potent dampening effects on key inflammatory mediators in human islets in vitro without any adverse effect on insulin secretion or viability. Although we have recently shown a potent anti-inflammatory effect by glucocorticoids added to human islets in vitro [27], the gold-standard immunosuppressive regimen in islet transplantation is glucocorticoid-free because of the diabetogenic effects of glucocorticoids [50]. This prompts the search for alternative anti-inflammatory strategies. Our data suggest a possible role for GW3965 as an anti-inflammatory agent in human islet culture prior to transplantation, and may represent a strategy to increase islet suitability for transplantation. However, lipotoxicity remains a concern, especially with high glucose concentrations and/or prolonged vigorous LXR stimulation. Even though LXR agonists hold promise in islet transplantation, the effects must be verified in vivo.

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