BBA - Molecular Basis of Disease 1865 (2019) 165525

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

Structure-toxicity relationships of saturated and unsaturated free fatty acids for elucidating the lipotoxic effects in human EndoC-βH1 beta-cells

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ARTICLE INFO

Keywords:
Human pancreatic beta cell
Lipotoxicity
Apoptosis
Fatty acid
ER stress
Type 2 diabetes

ABSTRACT

Lipotoxicity has been considered a major cause for beta-cell dysfunction in type 2 diabetes mellitus. However, the underlying mechanisms are still unclear. To achieve a better understanding of the toxicity a wide range of structurally different free fatty acids (FFAs) has been analyzed in human EndoC-βH1 beta-cells.

Exposure of human EndoC-βH1 beta-cells to physiological saturated and monounsaturated long-chain FFAs induced apoptosis. Particularly noteworthy was that the toxicity increased more rapidly with increasing chain length of saturated than of unsaturated FFAs. The highest toxicity was observed in the presence of very long-chain FFAs (C20-C22), whereas polyunsaturated FFAs were not toxic. Long-chain FFAs increased peroxisomal hydrogen peroxide generation slightly, while very long-chain FFAs increased hydrogen peroxide generation more potently in both peroxisomes and mitochondria. The greater toxicity of very long-chain FFAs was accompanied by hydroxyl radical formation, along with cardiolipin peroxidation and ATP depletion. Intriguingly, only saturated very long-chain FFAs activated ER stress. On the other hand saturated very long-chain FFAs did not induce lipid droplet formation in contrast to long-chain FFAs and unsaturated very long-chain FFAs.

The present data highlight the importance of structure-activity relationship analyses for the understanding of the mechanisms of lipotoxicity. Chain length and degree of saturation of FFAs are crucial factors for the toxicity of FFAs, with peroxisomal, mitochondrial, and ER stress representing the major pathogenic factors for induction of lipotoxicity. The results might provide a guide for the composition of a healthy beta-cell protective diet.

1. Introduction

Insulin resistance increases along with developing obesity causing hyperglycaemia and hyperlipidaemia during the development of type 2 diabetes mellitus. Initial compensatory hyperinsulinaemia tends to return stepwise to normoinsulinaemia and thereafter to hypoinsulinaemia while pancreatic beta-cell function gradually deteriorates [1] ending up in a fully developed open diabetic state. The decrease of insulin effectiveness and secretion is not only responsible for hyperglycaemia but also for the loss of suppression of lipolysis particularly in adipose tissue, explaining the strongly increased free fatty acid (FFA) concentration in the circulation in the diabetic state [2,3]. The cause for the deterioration of beta-cell function under a diabetic challenge is considered to be a combined gluco-lipotoxicity [4,5]. Potential mechanisms of lipotoxicity include oxidative stress, ER stress, and excessive accumulation of FFAs [4,6–11]. Studies on FFA toxicity in beta-cell lines of human origin have been restricted so far to the physiologically most abundant FFAs with chain lengths between C16 and C18 [12–14]. However, structure-activity relationships of physiological and non-physiological FFAs with respect to beta-cell lipotoxicity have not been studied in detail in human beta-cells. In the present study we therefore performed a detailed structure-toxicity analysis with a great variety of > 20 FFAs of different chemical structures to exploit the full potential of structure-toxicity relationships in the human EndoC-βH1 beta-cell line [15], allowing an identification of the underlying mechanisms of lipotoxicity. Therefore, we have investigated long-chain and very long-chain saturated and unsaturated, cis- or trans-configured FFAs, which are metabolized both mitochondrially and peroxisomally requiring a variable number of degradative beta-oxidation enzymes [16]. Thus, we focused in particular on the main FFA degradation pathways in mitochondrium and peroxisome as well as on the role of the ER stress in the development of lipotoxicity.

As a human beta-cell model the EndoC-βH1 beta-cell line was used in this study, since this cell line has proven to be a reliable surrogate for primary human beta-cells for physiological [1,17–20] and pathophysiological [20–22] studies. This human beta-cell line is equipped with

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https://doi.org/10.1016/j.bbadis.2019.08.001
Received 4 April 2019; Received in revised form 3 July 2019; Accepted 4 August 2019
Available online 06 August 2019

all the enzymes of the beta-oxidation pathways for metabolism of saturated and unsaturated FFAs [13].

As shown in the present study, this human beta-cell line in conjunction with the analysis of a knowledgeably selected wide range of FFAs with different chemical properties and metabolic fates proved to be a powerful tool for the elucidation of the complex mechanisms of lipotoxicity to human beta-cells. The present study shows that a number of mechanisms of FFAs contribute to the development of lipotoxicity and importantly that the role of the different mechanisms of lipotoxicity is not the same for each FFA. Rather the mechanisms of lipotoxicity differ in the case of saturated and unsaturated FFAs as well as long-chain (LC) and very long-chain (VLC) FFAs.

2. Materials and methods

2.1. Chemicals and materials

For FFA incubations a 50 mM stock solution of each FFA was freshly prepared using 90% ethanol as a solvent. FFAs were bound to fatty acid free bovine serum albumin (BSA) from Serva (Heidelberg, Germany) in low glucose (5.5 mM) DMEM (Dulbecco's Modified Eagle Medium) culture medium. The ratio between FFA and externally added BSA was 0.5 mM FFA to 1% BSA. For other FFA concentrations the BSA concentration was adapted according to this ratio. C16:0 (hexadecanoic acid/palmitic acid), C16:1 (cis-9-hexadecenoic acid/palmitoleic acid), C18:0 (octadecanoic acid/stearic acid), C18:1 (cis-9-octadecenoic acid/oleic acid), C18:1 (trans-9-octadecenoic acid/elaidic acid), C18:3 (cis-9,12,15-octadecatrienoic acid/α-linolenic acid), and C19:0 (nonadecanoic acid) were from Sigma-Aldrich (Munich, Germany).

C19:1 (nonadecenoic acid), C20:0 (eicosanoic acid/arachidic acid), C20:1 (cis-11-eicosenoic acid/gondoic acid), C20:2 (cis-11,14-eicosadienoic acid), C20:3 (cis-11,14,17-eicosatrienoic acid), C22:0 (docosanoic acid/behenic acid), C22:1 (cis-13-docosenoic acid/erucic acid), C22:2 (cis-13,16-docosadienoic acid), C22:3 (cis-13,16,19-docosatrienoic acid), and a variety of octadecenoic acids (ODAs) with a double bond at different positions (cis-6, cis-7, or cis-11) and the octadecadienoic acids (ODDAs) with two double bonds in different positions in cis or trans configuration (9,12 or 9,11) were all from Larodan AB (Solna, Sweden).

Cell culture medium consisted of DMEM low glucose medium (Life Technologies, Darmstadt, Germany), 2% BSA (Serologicals Proteins Inc., Kankakee, IL, USA), 100 μU/ml penicillin, 100 μg/ml streptomycin (Biochrom, Berlin, Germany), 50 μM 2-mercaptoethanol, 10 mM nicotinamide, 5 μg/ml transferrin, 6.7 ng/ml sodium selenite (all from Sigma-Aldrich). All other reagents were from Sigma-Aldrich or Merck unless stated otherwise.

2.2. Human EndoC-βH1 beta-cell line culture

EndoC-βH1 beta-cells (ENDOCELLS SARL, Paris, France) [15] were cultured onto coated dishes or multi-well plates in DMEM culture medium with 5.5 mM glucose, 2% BSA and without serum at 37 °C in a humidified atmosphere of 5% CO_2 as previously described [13,17,21]. The cells were routinely checked for mycoplasma and were tested free from mycoplasma contamination. Cells were cultured for one or two days in the presence of the different FFAs as described in the figure legends.

2.3. Caspase-3 activity assay

Human EndoC-βH1 beta-cells were seeded at a density of 30,000 cells/well onto coated white opaque 96-well plates two days before incubation with the indicated FFAs. Activation of caspase-3 was quantified by the CaspaseGlo-3/7 kit (Promega, Mannheim, Germany) according to the manufacturer's protocol. Data are expressed as percentages of untreated control cells.

2.4. Lipid droplet formation

Human EndoC-βH1 beta-cells were seeded at a density of 1.5×10^6 cells onto coated tissue culture dishes (Sarstedt, Nümbrecht, Germany) and cultured for three days before incubation with 500 μM of the indicated FFAs. Thereafter, the cells were trypsinized and fixed in 1% paraformaldehyde for 15 min at room temperature, followed by staining with 3.67 mM *Oil Red O* solution (Sigma-Aldrich) for 20 min at room temperature in the dark. After a PBS wash, lipid droplet formation was analyzed by fluorescence microscopy using the mCherry filter set (Olympus, Hamburg, Germany). Cells were analyzed as previously described [23].

2.5. Real-time quantitative reverse transcription PCR (qRT-PCR)

Total RNA from EndoC-βH1 beta-cells was isolated using the NucleoSpin RNA Plus kit (Macherey-Nagel, Düren, Germany) after exposure to the indicated FFAs. After quantification and quality control by NanoDrop 1000 (Thermo Fisher Scientific, Schwerte, Germany), 1–2 μg of RNA was reverse transcribed using random hexamer primers and RevertAid H Minus Reverse Transcriptase, which were obtained from Invitrogen (Karlsruhe, Germany). For qRT-PCR sequence-specific primers [21] and the GoTaq® qPCR Master Mix (Promega) were employed. Samples were first denatured at 95 °C for 2 min, followed by 40 PCR cycles, comprising a denaturation step at 95 °C for 15 s, an annealing step at 60 °C for 60 s, and an extension step at 72 °C for 30 s. Measurements were performed with the ViiA™ 7 Real-Time PCR System (Life Technologies, Darmstadt, Germany). The specificity of the amplified PCR product in each experiment was verified by melting curves. All measurements were performed in triplicates and normalized against the housekeeping genes β-actin, TATA-box binding protein, and α-tubulin using the qbasePLUS data analysis software (Biogazelle, Zulte, Belgium).

2.6. Generation of EndoC-βH1 beta-cells stably expressing HyPer H_2O_2 sensor proteins

To analyze the generation of H_2O_2 inside specific intracellular compartments, EndoC-βH1 beta-cells stably expressing the H_2O_2 sensor protein HyPer [24] inside the peroxisomes or the mitochondria were generated by lentiviral transduction.

For peroxisomal HyPer localization the peroxisomal targeting signal 1 (PTS-1) [25,26] was joined to the 3′-end of the HyPer cDNA by PCR using composite primers (HyPer-PTS1-*Eco*RI-fw 5′-TAGAATTCATGGAGATGGCAAGCCAGCAG-3′ and HyPer-PTS1-*Bam*HI-rv 5′-TAGGATCCTTACAGCTTGGAAACCGCCTGTTT-3′) and the pLenti6/V5-MCS-HyPer-Peroxi plasmid [27] as a template. The HyPer-Peroxi cDNA was inserted into the pLVX-EF1α-IRES-Puro plasmid via the *Eco*RI/*Bam*HI site.

For mitochondrial HyPer localization the HyPer cDNA with a double N-terminal mitochondrial targeting signal was amplified by PCR using composite primers (HyPer-Mito-*Eco*RI-fw 5′-TAGAATTCATGTCCGTCCTGACGCC-3′ and HyPer-Mito-*Xba*I-rv 5′-TATCTAGATTAAACCGCCTGTTTTAAAACTTTATC-3′) and the pHyPer-dMito plasmid (Evrogen, Moscow, Russia) as a template. Thereafter the HyPer-Mito cDNA was inserted into the pLVX-EF1α-IRES-Puro plasmid via the *Eco*RI/*Xba*I site.

For a stable expression of the peroxisomal and mitochondrial HyPer sensors in EndoC-βH1 beta-cells, lentiviruses were prepared as described before [28]. In brief, 5×10^6 293FT cells were transfected with the packaging plasmid pPAX2 (37.5 μg), the envelope plasmid pcDNA3-MDG (7.5 μg), and the respective transfer plasmids pLVX-EF1α-IRES-Puro-HyPer-Peroxi and pLVX-EF1α-IRES-Puro-HyPer-Mito (25 μg) by calcium phosphate precipitation. The virus particles were harvested from the culture medium 48 h later and purified in combination with sucrose solution by ultracentrifugation (25,000 rpm, 2 h at 4 °C). EndoC-βH1 beta-cells were infected with a virus dilution of 1:100 in the supernatant for 6 h. Cells were selected for expression of HyPer-Peroxi

or HyPer-Mito using puromycin (0.2 µl/ml).

2.7. Quantification of intracellular H_2O_2 by HyPer

Human EndoC-βH1 beta-cells stably expressing either the peroxisomally located H_2O_2-sensor HyPer (HyPer-Peroxi), or the mitochondrially located HyPer (HyPer-Mito) were seeded at a density of 1×10^5 cells onto coated black 24-well plates with coverglass bottom (Zellkontakt, Nörten-Hardenberg, Germany) and allowed to grow for three days. Thereafter, the HyPer expressing cells were exposed to the indicated FFAs at a concentration of 500 µM. Live cell imaging was performed using a CFP-YFP dual-band bandpass filter set (excitation: 436 nm and 500 nm; emission: 542 nm) with a cell^R/Olympus IX 81 inverted microscope system (Olympus). Xcellence rt software (Olympus) was used for imaging and analysis.

2.8. ATP-content

Human EndoC-βH1 beta-cells were seeded at a density of 8000 cells/well onto coated white opaque 96-well plates two days before incubation with the indicated FFAs. ATP-content was determined luminometrically (Promega) according to the manufacturer's protocol. Samples were measured on the GloMax-Multi Detection System (Promega). Data are expressed as percentages of untreated cells.

2.9. Cardiolipin peroxidation

Human EndoC-βH1 beta-cells were seeded at a density of 1×10^6 cells onto coated cell culture dishes (Greiner Bio-One, Frickenhausen, Germany). After two days of culture the cells were incubated with the indicated FFAs for another two days. Thereafter, EndoC-βH1 beta-cells were trypsinized and stained with nonyl acridine orange (acridine orange 10-nonyl bromide; NAO) as previously described [29].

2.10. Hydroxyl radical formation

Human EndoC-βH1 beta-cells were seeded at a density of 30,000 cells/well onto coated black 96-well plates two days before incubation with the indicated FFAs. Formation of hydroxyl radicals was measured by hydroxyphenyl fluorescein (HPF) fluorescence (Biomol, Hamburg, Germany). Therefore cells were pre-incubated with HPF for 30 min at a final concentration of 2 µM in Krebs-Ringer buffer without BSA. Data were normalized to a subsequent MTT cell viability test [30].

2.11. Western blot analysis

After incubation with the indicated FFAs human EndoC-βH1 beta-cells were trypsinized and collected in PBS (phosphate buffered saline). Cells were sonicated on ice for 15 s at 60 W. Protein content was determined by BCA assay (Thermo Fisher Scientific, Rockford, IL, USA). 30 µg of total protein/sample was separated by a 12.5% SDS-PAGE and electroblotted to a PVDF blotting membrane (GE Healthcare, Buckinghamshire, UK). The membranes were blocked with 5% nonfat dry milk in PBS with 0.1% Tween20 for 1 h at room temperature. After three washing steps with buffer (PBS, 0.1% Tween 20) the membranes were incubated with specific primary antibodies overnight at 4 °C (diluted in PBS, 0.1% Tween 20, 0.1% BSA). The utilized antibodies were: anti-cleaved caspase-3 (Asp175, diluted 1:500) from Cell Signaling (Beverly, MA, USA), anti-CHOP (15204, diluted 1:250) from Proteintech (Rosemont, IL, USA), anti-BIP (ab21685, diluted 1:500) from Abcam (Cambridge, UK), and β-actin (sc-47778, diluted 1:500) from Santa Cruz Biotechnology (Dallas, TX, USA). Then excess primary antibody was removed by washing with buffer. Afterwards, the membranes were incubated with a secondary anti-rabbit antibody at a dilution of 1:20,000 at room temperature for 1 h. Finally, protein bands were visualized by chemiluminescence using the ECL detection system (GE Healthcare). As a loading control the expression of β-actin was analyzed after stripping the blots with Reblot Plus Strong Solution (Merck) according to the manufacturer's manual with a secondary anti-mouse antibody. Protein bands were quantified and normalized to β-actin as IOD with Gel-Pro Analyzer (version 6.0, Media Cybernetics, Silver Spring, MD, USA).

2.12. Statistical analysis

Data are expressed as means ± SEM and statistical analyses were performed using one-way ANOVA plus Dunnett's test for multiple comparisons or Student's *t*-test (Graphpad Prism, San Diego, CA, USA), unless stated otherwise.

3. Results

3.1. Chain length dependent toxicity of long-chain (LC) and very long-chain (VLC) saturated and monounsaturated FFAs in human EndoC-βH1 beta-cells

Exposure of EndoC-βH1 beta-cells to LC and VLC FFAs induced a chain length-dependent (C16-C22) increase of the activation of the toxicity marker caspase-3 with increasing chain length (Fig. 1). Saturated and monounsaturated FFAs with the same chain length (C16:0, C16:1, C18:0, C18:1, C22:0, C22:1) showed a caspase-3 induction both on the protein expression level (Supplementary Fig. S1A) and the level of enzymatic activity (Fig. 1) after a two day incubation with a slight toxicity preference for the saturated FFAs. At variance, eicosanoic acid (C20:0) showed a > 3-fold higher toxicity than its monounsaturated counterpart eicosenoic acid (C20:1) (Fig. 1). Interestingly,

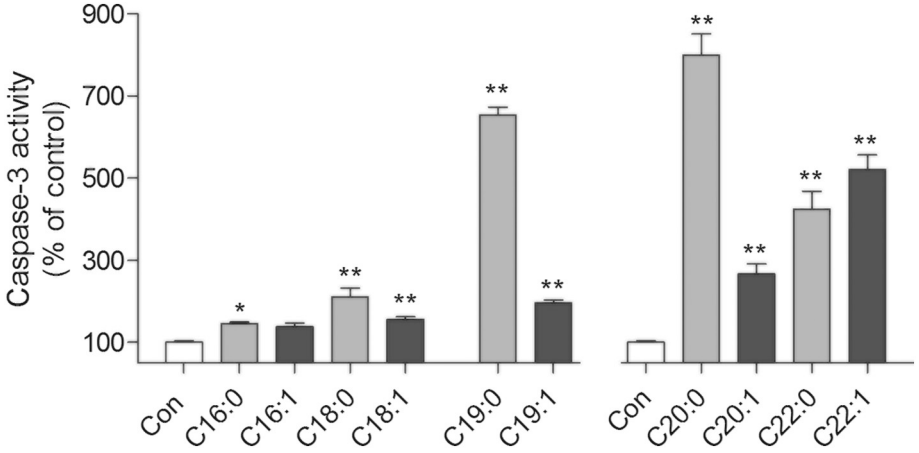

Fig. 1. Chain length dependency of the toxicity of saturated and monounsaturated long-chain (LC) and very long-chain (VLC) FFAs in human EndoC-βH1 beta-cells.
Human EndoC-βH1 beta-cells were incubated for two days with saturated and monounsaturated FFAs (C16-C22) (palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), nonadecanoic acid (C19:0), nonadecenoic acid (C19:1), arachidic acid (C20:0), gondoic acid (C20:1), behenic acid (C22:0), and erucic acid (C22:1)) (500 µM each). Thereafter, caspase-3 activity was measured. Data are given as means ± SEM of 5–9 independent experiments. *p < 0.05, **p < 0.01 compared to the respective untreated control cells (Dunnett's Multiple Comparison Test).

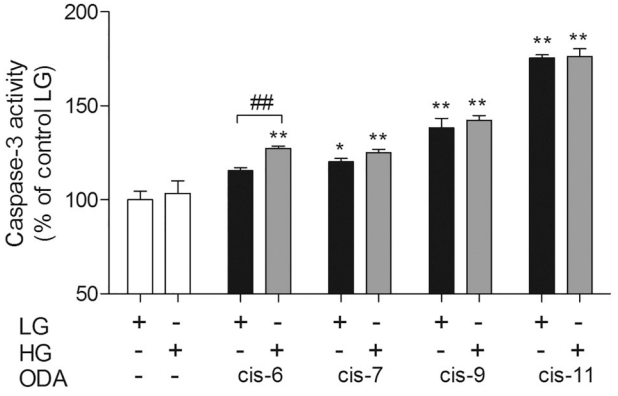

Fig. 2. Effects of different octadecenoic acids (ODAs) with a double bond at positions cis-6, cis-7, cis-9, or cis-11 in the presence of low glucose (LG) or high glucose (HG) on the toxicity to human EndoC-βH1 beta-cells.
Human EndoC-βH1 beta-cells were incubated for two days with different monounsaturated octadecenoic acids (ODAs with double bond at cis-6, cis-7, cis-9, or cis-11) (500 μM each) in the presence of low glucose (LG; 5.5 mM) or high glucose (HG; 25 mM). Thereafter, caspase-3 activity was measured. Data are given as means ± SEM of 5 independent experiments. *$p < 0.05$, **$p < 0.01$ compared to control LG or HG, respectively (Dunnett's multiple comparison test); ##$p < 0.01$ compared to the same condition with low glucose (LG) (Student's *t*-test).

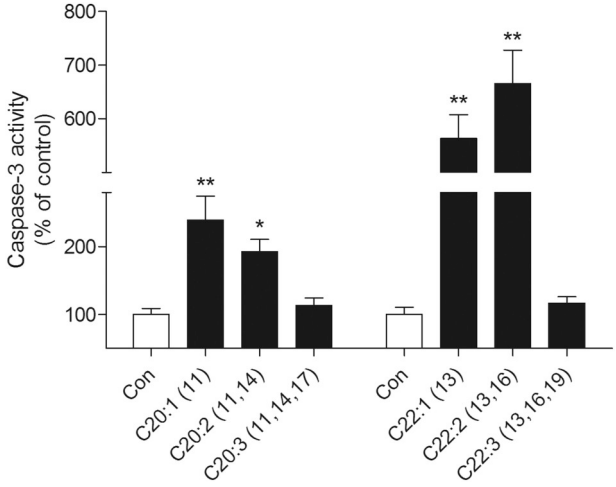

Fig. 3. Toxicity of very long-chain (VLC) eicosanoic acids (C20) and VLC docosanoic acids (C22) with an increasing number of double bonds in human EndoC-βH1 beta-cells.
Human EndoC-βH1 beta-cells were incubated for two days with various FFAs with a chain length of 20 or 22 C-atoms and an increasing number of double bonds (beginning with position cis-11 for C20 or with cis-13 for C22) (500 μM each). Thereafter, caspase-3 activity was measured. Data are given as means ± SEM of 4–5 independent experiments. *$p < 0.05$, **$p < 0.01$ compared to the respective untreated control cells (Dunnett's Multiple Comparison Test).

nonadecanoic acid (C19:0), a FFA with a chain length at the transition point between LC and VLC FFAs, showed toxicity to a comparable extent as the VLC FFAs C20:0 and C22:0, while nonadecenoic acid (C19:1) was still rather mildly toxic (Fig. 1).

3.2. Toxicity of different octadecenoic acids (ODAs) with one double bond in different positions and in the presence of 5.5 mM or 25 mM glucose in human EndoC-βH1 beta-cells

To analyze the influence of the double bond position in the acyl chain of the FFAs on caspase-3 activation, we tested various octadecenoic acids (ODAs) (C18:1) with one double bond at different positions. As shown in Fig. 2, a shift of the double bond within the ODAs from cis-6 to the positions cis-7, cis-9, or cis-11 showed a continuous increase in toxicity in the presence of a low glucose concentration (5.5 mM). At high glucose (25 mM) there was no significant toxicity increase for cis-7 ODA, cis-9 ODA and cis-11 ODA (Fig. 2). When comparing the ODAs with the position of the double bond in closest vicinity to the carboxyl group, namely cis-6 and cis-7 ODA, the toxicity was modestly but significantly increased at high glucose compared to low glucose only upon incubation with the even-numbered cis-6 ODA, but not with the odd-numbered cis-7 ODA (Fig. 2).

3.3. Toxicity of very long-chain (VLC) unsaturated eicosanoic acids (C20) and unsaturated docosanoic acids (C22) with an increasing number of double bonds in human EndoC-βH1 beta-cells

Next we determined the importance of the number of double bonds in the FFAs with respect to their toxicity. For this purpose, we analyzed the effect of C20 FFAs with one, two or three double bonds (Fig. 3). The first odd-numbered double bond was at position cis-11 (C20:1) (eicosenoic acid), the second additional even-numbered double bond at position cis-14 (C20:2) (eicosadienoic acid) and the third odd-numbered double bond at position cis-17 (C20:3) (eicosatrienoic acid) in the carbon chain. Incubation of beta-cells with C20:1 resulted in a significant toxicity. However, with each additional double bond the observed toxicity decreased in a stepwise manner, with a complete loss of toxicity when three double bonds were present in the fatty acid chain (Fig. 3).

In addition toxicity studies were performed with C22 FFAs with one, two, or three double bonds. The first odd-numbered double bond was located at position cis-13 (C22:1) (docosenoic acid), the second additional even-numbered double bond at position cis-16 (C22:2) (docosadienoic acid), and the third odd-numbered double bond at position cis-19 (C22:3) (docosatrienoic acid) in the carbon chain. Exposure of beta-cells to C22:1 or C22:2 caused severe toxicity, whereas C22:3 showed no toxicity compared to untreated control cells (Fig. 3).

3.4. Chain length-dependent peroxisomal and mitochondrial hydrogen peroxide (H_2O_2) generation in response to saturated and monounsaturated FFAs (C16-C22) in human EndoC-βH1 beta-cells

To investigate the relevance of metabolically generated H_2O_2 for FFA-mediated toxicity, we quantified the H_2O_2 generation in peroxisomes and mitochondria in the presence of various FFAs using the peroxisomally and mitochondrially located H_2O_2-specific HyPer sensors.

As shown in Fig. 4A, the physiological saturated (C16:0, C18:0) and monounsaturated LC FFAs (C16:1, C18:1) induced a low but significant production of H_2O_2 in peroxisomes. The most pronounced effects were observed with C19:0 and C19:1 (Fig. 4A). The VLC saturated (C20:0, C22:0) and monounsaturated FFAs (C20:1, C22:1) caused a markedly higher peroxisomal H_2O_2 production than the physiological FFAs (Fig. 4A).

However, LC FFAs (C16, C18) showed no significant increase of H_2O_2 generation in the mitochondria compared to untreated cells (Fig. 4B). C19:0 was the first FFA tested, which led to a significant mitochondrial H_2O_2 generation, comparable to the increase of the peroxisomal H_2O_2 generation. In contrast to physiological LC FFAs, all VLC FFAs induced a significant increase of H_2O_2 generation in the mitochondria (Fig. 4B).

The averaged levels of H_2O_2 generation of the four physiological LC FFAs (C16:0, C16:1, C18:0, C18:1) were slightly but significantly increased in the peroxisomes but not in the mitochondria; on the other hand, the averaged levels of H_2O_2 generation of the four VLC FFAs (C20:0, C20:1, C22:0, C22:1) showed a much more pronounced significant increase both in the peroxisomes and in the mitochondria

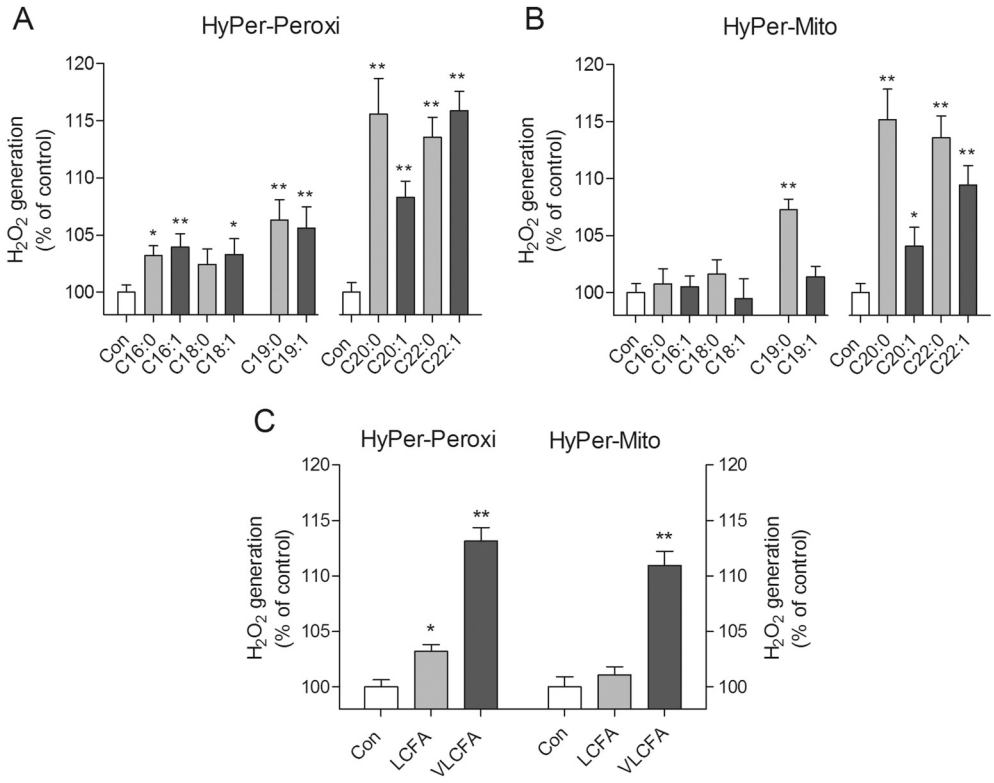

Fig. 4. H_2O_2 production after incubation with different saturated and monounsaturated long-chain (LC) and very long-chain (VLC) FFAs in human EndoC-βH1 beta-cells.
Human EndoC-βH1 beta-cells, which stably expressed the H_2O_2 sensor HyPer in peroxisomes (A) or mitochondria (B) were incubated with saturated and monounsaturated LC and VLC FFAs (C16-C22) (500 μM each) and H_2O_2 generation was measured. In addition, the averaged peroxisomal and mitochondrial H_2O_2 production in human EndoC-βH1 beta-cells upon exposure to the four saturated and monounsaturated LC FFAs (C16:0; C16:1; C18:0; C18:1) or to the four saturated and monounsaturated VLC FFAs (C20:0; C20:1; C22:0; C22:1) (500 μM each) is shown (C). The fluorescence ratio was measured after two days and normalized to the respective control cells. Data are given as means ± SEM of 10–15 independent experiments. *p < 0.05, **p < 0.01 compared to the respective control cells (Student's *t*-test).

(Fig. 4C). Thus, the metabolism of physiological FFAs was predominantly accompanied by peroxisomal H_2O_2 generation, whereas the metabolism of VLC FFAs went along with an additional mitochondrial H_2O_2 generation (Fig. 4C).

3.5. Chain length-dependent ER stress response to saturated and monounsaturated FFAs (C16-C22) in human EndoC-βH1 beta-cells

Next the induction of ER stress marker genes CHOP (also known as DDIT3), ATF4, and XBP1s in response to different FFAs was analyzed.

Incubation of beta-cells with saturated VLC eicosanoic and docosanoic acids (C20:0, C22:0) with a chain length > C18 caused a significant induction of CHOP both on the gene (Fig. 5B) and protein level (Supplementary Fig. S1B), as well as of ATF4 and XBP1s gene expression (Supplementary Fig. S2). The induction of these ER stress marker genes was also observed for nonadecanoic acid (C19:0), a FFA with a chain length at the transition between LC and VLC FFAs (Fig. 5A and Supplementary Fig. S2).

In contrast, saturated LC FFAs with a chain length < C19 had no significant effect on the investigated ER stress marker genes (Fig. 5A and Supplementary Fig. S2). All unsaturated FFAs did not induce the expression of the ER stress marker genes independent of their chain length (C16-C22) (Fig. 5A–B and Supplementary Fig. S2). This lack of influence of these fatty acids on CHOP expression could also be confirmed on protein level (Supplementary Fig. S1B).

Stearic acid (C18:0) was the only physiological FFA, which significantly induced the expression of the UPR genes BIP (also known as HSPA5) (Fig. 5C), GRP94 (also known as HSP90B1) as well as P58IPK (also known as DNAJC3) (Supplementary Fig. S2), all encoding for ER adaptive function. The gene expression of these three UPR genes was also significantly induced by nonadecanoic acid (C19:0) (Fig. 5A and Supplementary Fig. S2).

As shown in Fig. 5D and Supplementary Fig. S2 beta-cells showed a strong induction of the investigated UPR marker genes after incubation with saturated VLC FFAs (C20:0, C22:0). Interestingly, however, monounsaturated VLC FFAs (C20:1, C22:1) did not induce the gene expression of these UPR markers in the same way as the monounsaturated LC FFAs (C16:1, C18:1; C19:1) (Fig. 5C–D and Supplementary Fig. S2). This effect on BIP could be confirmed at the protein level (Supplementary Fig. S1B).

3.6. Induction of lipid droplet (LD) formation by different long-chain (LC) and very long-chain (VLC) saturated and monounsaturated (C16-C22) FFAs in human EndoC-βH1 beta-cells

Saturated LC (C16:0, C18:0, C19:0) and monounsaturated LC (C16:1, C18:1, C19:1) FFAs and monounsaturated VLC FFAs (C20:1, C22:1) (500 μM each) induced significant lipid droplet (LD) formation in beta-cells (Fig. 6). The increase of LDs was less pronounced with the LC FFAs (C16:0, C16:1, C18:0, C18:1) (around 10–15 fold) than with the two monounsaturated VLC FFAs (C20:1, C22:1) (around 32–44 fold). Nonadecanoic acid (C19:0) and nonadecenoic acid (C19:1) with a chain length in the transition zone caused the greatest increase of LD formation in the group of LC FFAs (around 20 fold). Lipid droplet formation was always somewhat higher for the unsaturated LC (C16:1-C19:1) than for the saturated LC (C16:0-C19:0) FFAs. In contrast, incubation of beta-cells with saturated VLC FFAs (C20:0, C22:0) showed no significant increase of LD formation (Fig. 6).

3.7. Effect of long-chain (LC) and very long-chain (VLC) saturated and monounsaturated FFAs on ATP-content, peroxidation of cardiolipin and formation of hydroxyl radicals in human EndoC-βH1 beta-cells

Along with increasing toxicity of FFAs (Fig. 1) hydroxyl radical formation and cardiolipin peroxidation increased while beta-cell ATP-content decreased (Fig. 7). Physiological LC FFAs with chain lengths of C16-C18 did not show significant effects (Fig. 7A–C). Nonadecanoic acid (C19:0) with a chain length in a transition zone was the first saturated FFA that decreased ATP-content (by around 50%) (Fig. 7A) and at the same time increased significantly cardiolipin peroxidation as well

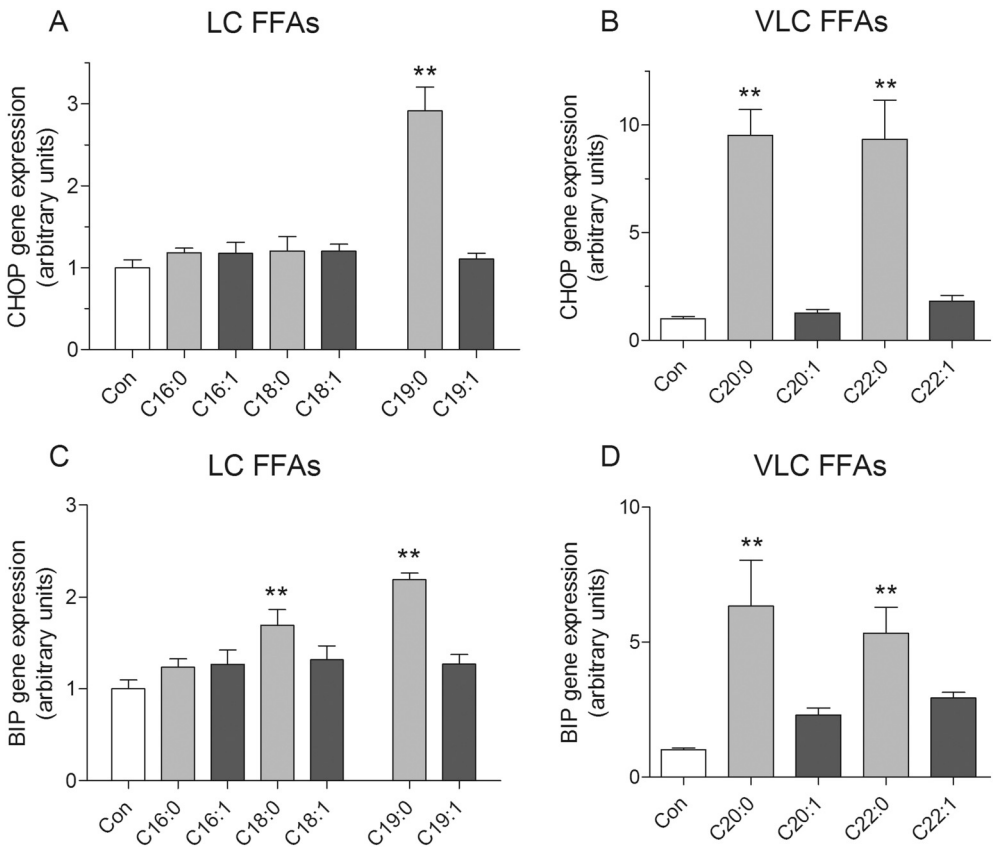

Fig. 5. Gene expression analysis of CHOP and BIP in response to different saturated and monounsaturated long-chain (LC) and very long-chain (VLC) FFAs (C16-C22) in human EndoC-βH1 beta-cells.
mRNA expression of the ER stress marker gene CHOP (A-B) and of the UPR (unfolded protein response) marker gene BIP (C–D) was quantified by qRT-PCR in human EndoC-βH1 beta-cells after a 24 h incubation with saturated and monounsaturated LC (A, C) and VLC (B, D) FFAs (C16-C22) (500 μM each). Expression levels were normalized to the expression of the reference genes actin, α-tubulin and the TATA box binding protein. Data are given as means ± SEM of 5–9 independent experiments. **p < 0.01 compared to the respective control cells (set 1) (Dunnett's Multiple Comparison Test).

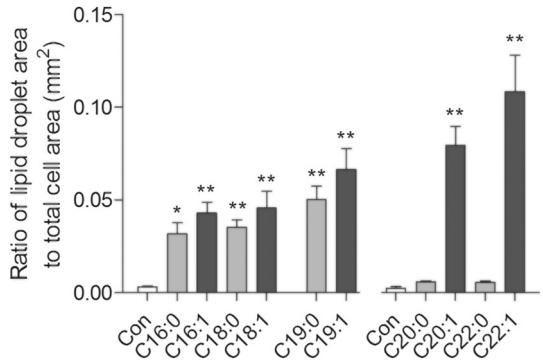

Fig. 6. Lipid droplet formation after incubation with different saturated and monounsaturated long-chain (LC) and very long-chain (VLC) FFAs in human EndoC-βH1 beta-cells.
Lipid droplet formation in human EndoC-βH1 beta-cells was determined after incubation with saturated and monounsaturated LC and VLC FFAs (C16-C22) (500 μM each) for two days. Thereafter, cells were fixed and stained with *Oil Red O*. To quantify lipid droplet formation in single cells, images were analyzed with the xcellence rt software. The fluorescence of lipid droplets is shown in relation to the total cell area and measured at 560/630 nm. Data are means ± SEM of 5–8 independent experiments. *p < 0.05, **p < 0.01 compared to the respective untreated control cells (ANOVA/Dunnett's Multiple Comparison Test).

as hydroxyl radical formation (Fig. 7B, C). In contrast, the monounsaturated FFA nonadecenoic acid (C19:1) did not affect these parameters (Fig. 7A–C). VLC saturated FFAs (C20:0, C22:0) decreased ATP-content even more (> 80%) (Fig. 7A) and in analogy increased markedly cardiolipin peroxidation (Fig. 7B) and hydroxyl radical formation (Fig. 7C). In contrast, the VLC unsaturated FFA C22:1 decreased ATP-content and increased hydroxyl radical formation, at variance from C20:1 (Fig. 7A, C) and both C20:1 and C22:1 increased cardiolipin peroxidation, even though to a lesser extent (Fig. 7B).

3.8. The modulatory role of double bonds in the chemical structure on the lipotoxic effects of FFAs on human EndoC-βH1 beta-cells: a comparison between physiological and non-physiological unsaturated FFAs

In order to further elucidate the mechanism of beta-cell lipotoxicity we decided to analyze in a final step in this long sequence of experimentation the toxic potential of a variety of octadecadienoic acids (ODDAs) in cis and trans configuration with double bonds in different positions in the carbon chain. These were on the one hand physiological FFAs and on the other hand non-physiological FFA model substances. We selected these FFAs with respect to their different metabolic fate in the beta-oxidation. These results were compared with those obtained with the two octadecenoic acids (ODAs) (C18:1) in cis and trans configuration. This makes it possible to identify the structural requirements of the various FFAs responsible for their differential lipotoxic potency in dependence upon their metabolic fate in the peroxisomal and mitochondrial beta-oxidation pathways.

The monounsaturated ODAs (C18:1) in cis and trans configuration with a double bond in position 9 in the carbon chain (cis-9 and trans-9) were both moderately beta-cell toxic (Table 1). The diunsaturated ODDAs (C18:2) in cis and trans configuration with double bonds in positions 9 and 12 in the carbon chain (cis-9,12 and trans-9,12) showed a comparable toxicity (Table 1).

In contrast, the diunsaturated ODDA in trans configuration with odd-numbered double bonds in positions 9 and 11 in the carbon chain (trans-9,11) was by far the most toxic species within ODDAs tested in the present study (Table 1).

At variance, the cis-configured ODDA with odd-numbered double bonds in positions 9 and 11 in the carbon chain (cis-9,11) was not toxic at the same concentration (Table 1). And interestingly, replacement of the trans-configured double bond in position 9 of trans-9,11 by a cis-configured double bond (cis-9,trans-11) abolished the increase of the

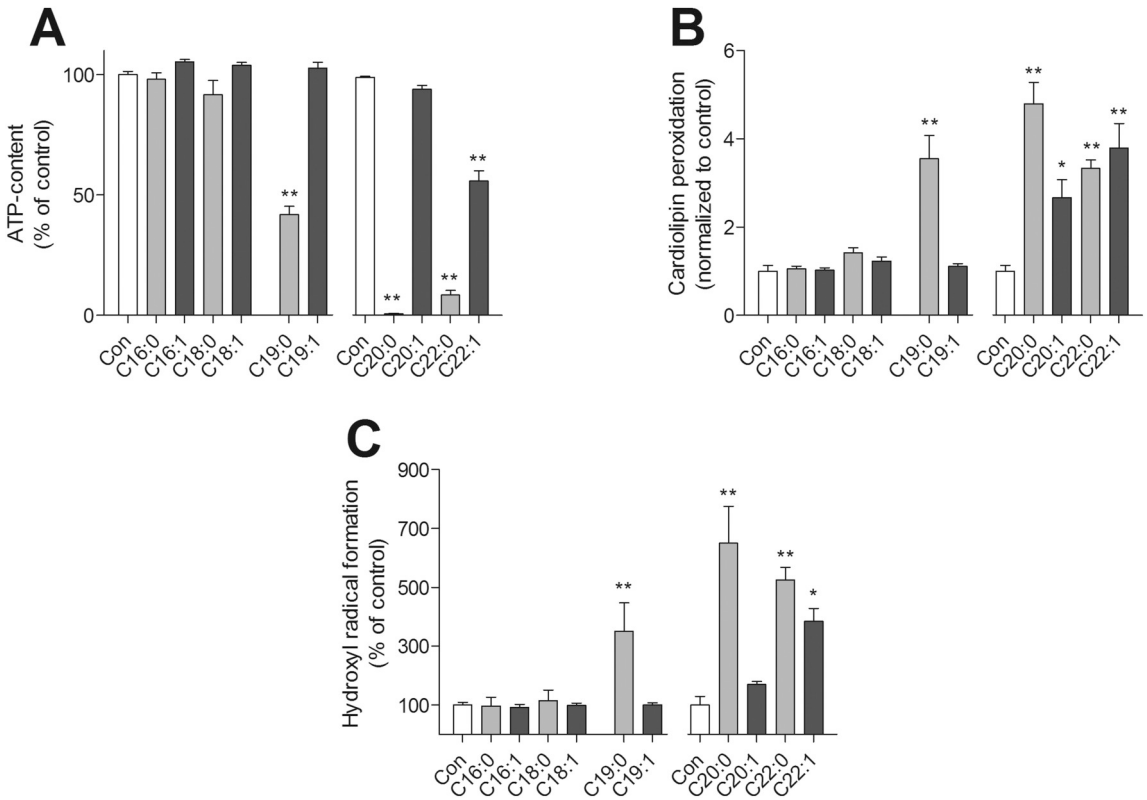

Fig. 7. Effects of saturated and monounsaturated long-chain (LC) and very long-chain (VLC) FFAs on the ATP-content, peroxidation of cardiolipin and hydroxyl radical formation in human EndoC-βH1 beta-cells.

Human EndoC-βH1 beta-cells were incubated for two days with saturated and monounsaturated LC and VLC FFAs (C16-C22) (500 μM each). Thereafter, ATP-content (A) was measured luminometrically and cardiolipin peroxidation (B) was measured fluorimetrically. Hydroxyl radical formation (C) was determined by the fluorescence change of the dye HPF (hydroxyphenyl fluorescein) and normalized to the results of a subsequent MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) viability test. Data are given as means ± SEM of 4–6 independent experiments. *$p < 0.05$, **$p < 0.01$ compared to the respective untreated control cells (Dunnett's Multiple Comparison Test).

caspase-3 activity nearly completely (Table 1).

All FFAs in Table 1 increased LD formation, most potently the diunsaturated ODDA trans-9,11. Out of all the different FFAs in Table 1 the trans-9,11 ODDA increased the H_2O_2 formation most strongly in the peroxisomes and in addition was the only FFA that significantly increased also mitochondrial H_2O_2 formation (Table 1). The increases of H_2O_2 formation in these two subcellular organelles by the trans-9,11 ODDA went along with severe toxicity, not only documented by a very pronounced increase of caspase-3 activity but also by a very strong decrease of ATP content and a very strong increase of cardiolipin

Table 1

Effect of different octadecadienoic acids (ODDAs) (C18:2) in comparison with two octadecenoic acids (ODAs) (C18:1) with different cis- or trans-configured double bonds with respect to the toxicity to human EndoC-βH1 beta-cells.

Human EndoC-βH1 beta-cells were incubated for two days with different physiological and non-physiological mono-, and diunsaturated octadecanoic acids with double bonds at different positions. Thereafter, caspase-3 activity, H_2O_2 generation, CHOP and BIP gene expression, lipid droplet (LD) formation, ATP-content and cardiolipin peroxidation were measured in comparison to the respective control. Data are expressed as % of control and means ± SEM of the numbers of experiments given in parentheses. *$p < 0.05$, **$p < 0.01$ compared to the respective controls (set 100%) (Student's *t*-test and Dunnett's Multiple Comparison Test).

ODA/ODDA (double bond position and configuration)	Caspase-3 activity (%)	HyPer-Peroxi fluorescence (%)	HyPer-Mito fluorescence (%)	ER stress: CHOP-expression (%)	UPR: BIP-expression (%)	LD formation(%)	ATP-content (%)	Cardiolipin peroxidation (%)
cis-9	156 ± 6 *(6)	103.3 ± 1.4 *(15)	99.5 ± 1.7 (10)	115 ± 11 (12)	132 ± 15 (6)	1090 ± 217 **(5)	104 ± 1 (4)	114 ± 3 (4)
trans-9	166 ± 8 *(4)	105.0 ± 0.8 **(9)	101.6 ± 1.6 (8)	101 ± 13 (7)	124 ± 8 (6)	1246 ± 166 **(7)	102 ± 2 (4)	104 ± 2 (5)
cis-9,12	164 ± 11 *(4)	104.6 ± 1.3 *(4)	100.6 ± 1.0 (4)	129 ± 8 (8)	125 ± 9 (4)	1053 ± 122 **(8)	101 ± 1 (6)	101 ± 2 (4)
trans-9,12	171 ± 10 **(4)	105.5 ± 1.2 **(4)	100.0 ± 0.5 (4)	111 ± 7 (8)	118 ± 6 (4)	1039 ± 169 **(6)	103 ± 1 (6)	120 ± 2 (4)
cis-9,11	139 ± 5 (4)	107.4 ± 0.8 **(10)	101.4 ± 0.9 (10)	104 ± 8 (8)	92 ± 12 (6)	803 ± 150 **(9)	93 ± 1 (4)	111 ± 5 (5)
cis-9,trans-11	122 ± 12 (4)	103.8 ± 1.0 *(7)	99.5 ± 1.1 (6)	98 ± 7 (4)	105 ± 12 (4)	427 ± 17 (4)	102 ± 2 (4)	114 ± 4 (4)
trans-9,11	478 ± 36 **(5)	110.9 ± 0.8 **(10)	105.7 ± 1.5 **(10)	1797 ± 249 **(8)	248 ± 38 **(6)	1342 ± 268 **(9)	16 ± 3 **(4)	492 ± 84 **(5)

peroxidation as clear indicators of beta-cell death through mitochondrial damage (Table 1). Remarkable is also the fact that the toxicity of trans-9,11 ODDA was accompanied by an enormous increase of the expression of the UPR marker BIP and the ER stress marker CHOP (Table 1).

In summary, the FFAs with double bonds in cis or trans configuration in position 9 as well as with two double bonds in positions 9 and 12 were only moderately toxic and this moderate toxicity was accompanied only by minor effects on peroxisomal and mitochondrial H_2O_2 formation as well as on ATP-content, cardiolipin peroxidation and on UPR and ER stress marker expression (Table 1). The trans-9,11 ODDA with its strong toxicity marked the other end of the toxicity spectrum (Table 1).

4. Discussion

Saturated as well as monounsaturated and diunsaturated long-chain (LC) FFAs (C16-C18) and even more so very long-chain (VLC) FFAs (C20-C22) were toxic to human EndoC-βH1 beta-cells. Since an analogous toxic potential of physiological saturated and unsaturated FFAs has been reported previously also for beta-cells in rat and human islets [13] the EndoC-βH1 beta-cells represent such an ideal tool for the present extensive structure-toxicity analyses. Caspase-3 induction was comparable for the respective saturated and monounsaturated LC FFAs with the same chain length, but increased more rapidly with increasing chain length in the case of the saturated FFAs than the unsaturated FFAs. This preference for saturated FFAs was also observed in studies with another human beta-cell line [14].

In general, double bonds retard the metabolic flux rate [31–34] and hamper the generation of H_2O_2 in the beta-oxidation pathways. The reduction of the toxicity was as more pronounced as more proximal the double bond was positioned to the carbonyl group, since this reduced the number of undisturbed cycles in the beta-oxidation spiral. It is therefore not surprising that toxicity decreased in the case of the odd-numbered monounsaturated octadecenoic acid (ODA) isomers from cis-11 ODA > cis-9 ODA > cis-7 ODA in parallel with a reduction of the number of cycles in the beta-oxidation from four to three to two, before the double bond in the fatty acid chain is reached.

No significant increase of toxicity was observed in the case of the odd-numbered cis-7, cis-9, and cis-11 ODA isomers, when the glucose concentration in the incubation medium was raised from a low value of 5.5 mM to a high value of 25 mM. Interestingly, however, there was a slight but significant increase of the toxicity under high glucose in the case of the even-numbered cis-6 ODA isomer. The effect is apparently related to the dependence on the reduced form of nicotinamide adenine nucleotide phosphate (NADPH) in the metabolic step catalyzed by the enzyme 2,4-dienoyl-CoA reductase in the breakdown of double bonds in even-numbered unsaturated FFAs [33]. This significant increase can be explained by the greater availability of NADPH in the presence of a high glucose concentration, supporting the more efficient regeneration of the oxidized $NADP^+$ required as a coenzyme in this metabolic step, through reduction in the pentose phosphate pathway [4]. The additional increase in toxicity induced by glucose in the case of this even-numbered cis-6 ODA isomer is moderate, compared with the toxicity of the FFA as such. This is in agreement with the fact that the glucose component in the gluco-lipotoxicity mediated by reactive oxygen species, especially in the case of H_2O_2-mediated toxicity, is limited when compared with the lipid component [4].

Furthermore, the toxicity decreased along with an increase of the number of double bonds in the FFA. This was documented in a comparison of the toxicity of the VLC eicosanoic acids (C20) with one, two or three double bonds as well as of the VLC docosanoic acids (C22) with one, two or three double bonds. A change in the catabolism from odd-numbered to even-numbered beta-oxidation mechanisms of these VLC FFAs during chain-shortening, as it is typical for polyunsaturated FFAs [33–37], reduced toxicity not only due to the increasing number of double bonds but additionally also due to alternating changes between odd-numbered and even-numbered double bond chain-shortening mechanisms in the beta-oxidation of polyunsaturated FFAs [33–37]. Three double bonds in the carbon chain as in the case of C20:3 went along with a complete loss of toxicity. The same was true for C22:3. This phenomenon was observed even though to a lesser extent also with the increasing number of double bonds in the case of the physiological octadecanoic acids (C18) with one, two or three double bonds [13].

Mitochondrial damage documented by ATP depletion and cardiolipin peroxidation was not caused by physiological FFAs, but became evident at a chain length of 19 carbon atoms and was more pronounced with saturated FFAs than with unsaturated FFAs. This is in line with previous results [38]. The ultimate reason for the loss of the structural and functional integrity of the mitochondria through FFAs was the hydroxyl radical formation. Overall, mitochondrial impairment started at an earlier chain length with saturated FFAs (C19:0) when compared to monounsaturated FFAs (C20:1).

Both the moderately toxic saturated and monounsaturated LC FFAs with a chain length ≥ C16 (C16-C18) induced also moderate lipid droplet (LD) formation in beta-cells. LD formation has been considered to be a potential mechanism of sequestration of FFAs into membrane-coated intracellular vesicles [39]. It could thus theoretically represent a mechanism to reduce toxicity of FFAs [40–42]. In fact, LD formation increased in EndoC-βH1 beta-cells along with an increasing chain length in parallel to beta-cell toxicity as shown in the case of the monounsaturated VLC FFAs (C20:1, C22:1). Interestingly, this strong LD formation went along with an inability of the unsaturated VLC FFAs to induce ER stress. On the other hand, saturated VLC FFAs caused an increased ER stress response in the absence of LD formation, possibly due to the fact, that these FFAs may be poor substrates for acyl transferases. Thus, sequestration into lipid droplets may not be the sole mechanism for prevention of FFA toxicity in beta-cells even if there is an overall tendency for a reduction of FFA-induced ER stress.

The VLC saturated FFAs (C20:0, C22:0) caused a significant induction of both UPR genes, encoding for ER adaptive function, and pro-apoptotic ER stress genes. In contrast, neither LC saturated and monounsaturated FFAs nor VLC monounsaturated FFAs had an effect on the gene expression of the ER stress or UPR markers. Interestingly, stearic acid (C18:0), the saturated LC FFA with the longest carbon chain among the physiological FFAs, induced adaptive UPR encoding genes but no ER stress marker genes. This chain length dependent move towards ER stress is further supported by the observation that the odd-numbered nonadecanoic acid (C19:0) with a chain length in the transition zone between LC and VLC FFAs induced both UPR and ER stress marker genes. So stearic acid (C18:0) had exactly the chain length, which might still enabled suppression of maladaptive ER stress through increased expression of protective chaperones, while nonadecanoic acid (C19:0) could no more achieve this goal. The results with stearic acid document that it is in principle possible to prevent a transition into an apoptotic ER stress. On the other hand, toxicity of unsaturated FFAs is not dependent upon an activation of ER stress genes in beta-cells.

It can therefore be concluded, that the beta-cell toxicity profiles of the different FFA species can differ very much and that there is no single FFA with a toxicity profile representative for all FFAs. In principle four groups of FFAs can be distinguished and there are prototype FFAs, which are representative for each of these groups. A number of key messages for the understanding of the pathology of FFAs in human EndoC-βH1 beta-cells can be derived from these different groups.

1) Toxic physiological saturated (C16:0, C18:0) and monounsaturated (C16:1, C18:1) as well as diunsaturated (C18:2) LC FFAs with LD formation and peroxisomal H_2O_2 generation, but without significant ER stress and without mitochondrial impairment (prototypes: palmitic acid (C16:0) and oleic acid (C18:1)).
2) Highly toxic saturated VLC FFAs with ER stress and both peroxisomal and in addition mitochondrial H_2O_2 generation, but without

LD formation (prototypes: arachidic acid (C20:0) and behenic acid (C22:0)).
3) Toxic monounsaturated VLC FFAs with LD formation and both peroxisomal and mitochondrial H_2O_2 generation, but without induction of ER stress marker genes (prototypes: gondoic acid (C20:1) and erucic acid (C22:1)).
4) Polyunsaturated FFAs with very low beta-cell toxicity, H_2O_2 generation, and LD formation (prototype: linolenic acid (C18:3)).
5) Overall, the toxicity of saturated FFAs increased more quickly with increasing chain length than it was the case for the toxicity of unsaturated FFAs. This was in particular visible in the cases of the FFAs with 19 and 20 carbon atoms. This faster development of toxicity along with an increasing chain length in the case of the saturated FFAs as compared to the monounsaturated FFAs correlates with the fact that only the saturated but not unsaturated FFAs cause a stepwise increase of ER stress gene expression.

In a last step we compared the beta-cell toxic potential of various unsaturated octadecadienoic acids (ODDAs) with odd- and even-numbered double bonds in positions 9 or 9 and 12 or with only odd-numbered double bonds in positions 9 or 9 and 11. The degradation of these special non-physiological model ODDAs differs from the physiological unsaturated ODDAs in that they do not fully depend on the complex enzymatic equipment of the beta-oxidation. Metabolic degradation is therefore less complex and more straight forward. This is true specifically for the ODDAs in the non-physiological trans configuration. Monounsaturated ODAs with one double bond in position 9 and ODDAs with two double bonds in positions 9 and 12 in the trans configuration tended to be slightly more toxic than in the cis configuration. This higher toxicity of FFAs with double bonds in the trans configuration is consistent with results from previous studies on human beta-cell lines [12].

In contrast, the diunsaturated ODDA (C18:2) with odd-numbered double bonds in positions 9 and 11 in the carbon chain in trans configuration (trans-9,11) was extremely toxic. It was the by far most toxic species within a wide range of ODDAs tested in the present study. The replacement of the trans-configured double bond in position 9 of trans-9,11 by a cis-configured double bond (cis-9,trans-11) reduced caspase-3 activity drastically approaching nearly control values. The extreme toxicity of this diunsaturated model ODDA (C18:2) in trans configuration (trans-9,11) in contrast to the diunsaturated model ODDA (C18:2) in cis configuration (cis-9,11) can be explained by the fact that the enzyme enoyl-CoA isomerase is not needed any more for the transformation into the trans configuration in this distal step of the beta-oxidation [34] thereby accelerating the metabolic rate.

All ODDAs increased lipid droplet (LD) formation, most strongly again the diunsaturated trans-9,11 ODDA. Out of all the different ODDAs in Table 1 the trans-9,11 ODDA increased the H_2O_2 formation most strongly in the peroxisomes and in addition was the only ODDA that significantly increased mitochondrial H_2O_2 formation. The increases of H_2O_2 formation in these two subcellular organelles by the trans-9,11 ODDA went along with strong toxicity, not only documented by a very pronounced increase of caspase-3 but also by a very strong decrease of ATP-content and an increase of cardiolipin peroxidation as clear indicators of beta-cell death through mitochondrial damage (Table 1). Remarkable is also the fact that the toxicity of trans-9,11 ODDA was accompanied by an enormous increase of the expression of the ER stress marker CHOP and the UPR marker BIP. Thus, trans-9,11 ODDA was the only FFA, which combined LD formation and an enormous increase of the ER stress gene expression (Table 1).

5. Conclusion

The results document the crucial importance of FFA metabolism for beta-cell lipotoxicity with peroxisomal stress, mitochondrial stress and ER stress representing the major components. Moderate toxicity of the physiological LC FFAs was linked to peroxisomal stress, high toxicity of VLC FFAs was linked in addition to mitochondrial stress and in the case of the very high toxicity of saturated VLC FFAs went along with pronounced ER stress. The present experiments indicate that it might be sensible to restrict the lipid component of our diet to physiological FFAs in cis configuration. This prevents excessive toxicity to the beta-cells, as long as overnutrition and an overload of the adipose tissue with FFAs are avoided [43–45]. The complex metabolism in particular of the polyunsaturated FFAs with alternating odd- and even-numbered double bonds retards metabolic flux through the peroxisomal and mitochondrial beta-oxidation pathways and thereby reduces the generation of toxic reactive oxygen species. At the same time the results provide an argument for a ban of VLC FFAs and of trans-configured FFAs from our diet, when aiming at a protection against lipotoxicity. In conclusion the results of the present study offer a guide for an optimal composition of the lipid component of the daily diet with respect to a protection of pancreatic beta-cells and thus a prevention or at least an attenuation of a deterioration of glucose tolerance triggered by FFAs.

Transparency document

The Transparency document associated with this article can be found in online version.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the European Union (Collaborative Project BetaBAT in the Framework Programme 7, grant agreement 277713). T.P. and A.L. have been PhD students within the Research Training Group (RTG) 1947 BiOx of the German Research Council at the University of Greifswald.

Author contribution

TP designed and performed experiments, analyzed data, and created figures and tables and co-supervised the project; BK, ASvH, AL performed experiments and analyzed data; IM coordinated and analyzed ER stress experiments; SL designed experiments, and supervised and coordinated the project. TP, IM and SL wrote the manuscript and all other authors revised and approved the final version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2019.08.001.

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