Peroxisome generated hydrogen peroxide as important mediator of lipotoxicity in insulin-producing cells

Running title: Hydrogen peroxide mediated lipotoxicity

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Submitted 21 September 2009 and accepted 5 October 2010.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org

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Objective: Type 2 diabetes mellitus is a complex disease that is accompanied by elevated levels of non-esterified fatty acids (NEFAs), which contribute to β-cell dysfunction and β-cell loss, referred to as lipotoxicity. Experimental evidence suggests that oxidative stress is involved in lipotoxicity. In this study, we analyzed the molecular mechanisms of reactive oxygen species-mediated lipotoxicity in insulin-producing RINm5F cells and INS-1E cells as well as in primary rat islet cells.

Research Design and Methods: The toxicity of saturated NEFAs with different chain lengths upon insulin-producing cells was determined by MTT and PI viability assays. Catalase or superoxide dismutase overexpressing cells were used to analyze the nature and the cellular compartment of reactive oxygen species formation. With the new H$_2$O$_2$-sensitive fluorescent protein HyPer H$_2$O$_2$ formation induced by exposure to palmitic acid was determined.

Results: Only long chain (>C14) saturated NEFAs were toxic to insulin-producing cells. Overexpression of catalase in the peroxisomes and in the cytosol, but not in the mitochondria, significantly reduced H$_2$O$_2$ formation and protected the cells against palmitic acid-induced toxicity. With the HyPer protein H$_2$O$_2$ generation was directly detectable in the peroxisomes of RINm5F and INS-1E insulin-producing cells as well as in primary rat islet cells.

Conclusions: The results demonstrate that H$_2$O$_2$ formation in the peroxisomes rather than in the mitochondria are responsible for NEFA-induced toxicity. Therefore, we propose a new concept of fatty acid-induced β-cell lipotoxicity mediated via reactive oxygen species formation through peroxisomal β-oxidation.
Hydrogen peroxide mediated lipotoxicity

by enhancing formation of reactive oxygen species (ROS) in the mitochondria (15). Superoxide radicals are generated at complexes I and III of the respiratory chain (22) and can give rise to toxic hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (23; 24). Interestingly, some studies have suggested that mitochondrial β-oxidation can be protective, while inhibition of β-oxidation increases lipotoxicity (25; 26). However, neither concept fully explains the molecular mechanism of lipotoxicity.

Herein, we provide experimental evidence in support of an entirely new concept of NEFA-induced β-cell lipotoxicity based on peroxisomal metabolism of NEFAs. Long-chain NEFAs, such as palmitic and stearic acid, can be metabolized through β-oxidation in the peroxisomes as well as in the mitochondria (27; 28). In contrast to mitochondrial β-oxidation, the acyl-CoA oxidases in the peroxisomes form H$_2$O$_2$ and not reducing equivalents (28). For H$_2$O$_2$ inactivation, the oxidoreductase catalase is typically expressed in peroxisomes (28). However, expression of H$_2$O$_2$-inactivating catalase is virtually absent in the peroxisomes of insulin-producing cells (29; 30). This lack of a low affinity, high capacity H$_2$O$_2$-inactivating enzyme (29; 30) impedes inactivation of peroxisome-generated H$_2$O$_2$, thereby increasing the vulnerability of pancreatic β-cells to ROS-mediated lipotoxicity (15; 23).

RESEARCH DESIGN AND METHODS

Tissue culture of insulin-producing cells. Insulin-producing RINm5F cells and INS-1E cells (kindly provided by C. Wollheim) were cultured as described previously (30; 31).

RINm5F cell clones that overexpressed different antioxidative enzymes were generated as described previously (32; 33). Cellular expression of antioxidative enzymes was analyzed by Western blot or catalase enzyme activity measurement (33).

For the quantification of the catalase activity the method originally described by (34) was used. In brief, cells were homogenized in phosphate buffered saline (pH 7.4) through sonication on ice for 1 min in 15-s bursts at 90 W. Catalase activity was measured by ultraviolet spectroscopy, monitoring the decomposition of H$_2$O$_2$ at 240 nm. Catalase activity was calculated by the following equation:

\[
U / mg = \frac{\Delta A \cdot min^{-1} \cdot 1000 \cdot ml \, Reaction \, Mix}{43.6 \cdot mg \, Protein}
\]

NEFAs (Sigma, St. Louis, MO, USA) were dissolved in 90% ethanol heated to 60°C and used at different concentrations in RPMI 1640 (PAN, Aidenbach, Germany) with 1% fetal calf serum and a final BSA (MP Biomedicals, Eschwege, Germany) : NEFA ratio of 2% : 1 mM, according to (13). All control wells received the same amount of solvent and BSA. This procedure did not cause a significant decrease in viability in the absence of added fatty acids.

Rat islet isolation and culture. Pancreatic islets were isolated from 250-300 g adult male Lewis rats by collagenase digestion, separated by Ficoll gradient, and handpicked under a stereo microscope. Isolated islets were cultured on extracellular matrix (ECM)-coated plates (35mm) (Novamed, Jerusalem, Israel, the ECM being derived from bovine corneal endothelial cells) in RPMI-1640 medium containing 5 mM glucose, 10% FCS, penicillin, and streptomycin at 37 °C in a humidified atmosphere of 5% CO$_2$ according to (35) et al. The islets were cultured for 7-10 d on the ECM plates to adhere and spread before they were infected with HyPer-Peroxi lentivirus or treated with palmitic acid.

Assessment of cell viability. RINm5F mock-transfected and cytoprotective enzyme overexpressing RINm5F insulin-producing cells were seeded at 25,000 cells/well in 100 µl culture medium in 96-well plates (for PI staining, black 96-well plates) and allowed to attach for 24 h before they were incubated at
Hydrogen peroxide mediated lipotoxicity

37°C with NEFAs for 24 h. Cell viability was then determined by either microplate-based MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (36) or PI staining (Sigma, St. Louis, MO, USA). PI is membrane impermeant and usually excluded from viable cells (37). To measure the rate of cell death after NEFA treatment, the PI stock solution (12.5 µg/ml) was diluted in the incubation media to a final concentration of 0.625 µg/ml. After 15 min incubation in the dark, the plates were analyzed at 520/620 nm excitation/emission using the fluorescence reader Victor 2 1420 Multilabel Counter (Perkin Elmer, Wiesbaden, Germany).

**Determination of oxidative stress by DCF-DA fluorescence.** To detect overall oxidative stress, 25,000 cells were seeded in 96-well black plates and cultured for 24 h. The cells were then pre-incubated with 10 µM DCF-DA (2′, 7-dichlorofluorescein diacetate, Sigma, St. Louis, MO, USA) for 30 min at 37°C. Thereafter, medium containing DCF-DA was replaced with fresh medium with or without palmitic acid. After 24 h incubation, the plates were analyzed at 480/520 nm excitation/emission using the fluorescence reader Victor 2 1420 Multilabel Counter (Perkin Elmer, Wiesbaden, Germany) (38). Data are expressed as percentages relative to untreated cells.

**Cloning of HyPer vectors.** For in vivo analyses of mitochondrial H₂O₂ generation, the cDNA of the H₂O₂-sensitive fluorescent HyPer protein (39) was subcloned from the pHyPer-dMito (Evrogen, Moscow, Russia) into the lentiviral transfer plasmid pLenti6/V5-MCS (Invitrogen, Karlsruhe, Germany). The cDNA was excised from the pHyPer-dMito plasmid using the NheI and NotI restriction sites, blunted, and ligated into the EcoRV site of the pLenti6/V5-MCS plasmid. To construct the expression vector for the HyPer-Peroxi protein the peroxisome target signal (PTS1) (40; 41) was joined to the 3′-end of the HyPer cDNA by PCR using composite Primer (HyPer-PTS1-XbaI-fw (5′-TATCTAGACGCCCACCATGGAGATGGCA A-3′) and HyPer-PTS1-Bsp119I-rv (5′-GCTTCGAAATTACAGCTTGGAAACCGCC TGTITTAAAC-3′)) and the pHyper-dCyto plasmid as template. Then, the HyPer-Peroxi cDNA was subcloned into the XbaI/Bsp119I site of the pLenti6/V5-MCS plasmid.

**Preparation of lentiviruses.** To express the HyPer-Mito and HyPer-Peroxi proteins lentivirus was prepared according to (42): 500,000 293FT cells were transfected with the packaging plasmid pPAX2 (37.5 µg), the envelope plasmid pcDNA-MDG (7.5 µg), and the transfer plasmids pLenti6/V5-MCS-HyPer-Mito or pLenti6/V5-MCS-HyPer-Peroxi (25 µg) by calcium phosphate precipitation. The virus particles were harvested from the culture medium 48 h later and purified by ultracentrifugation (2 h at 70,000 g). The virus titers (3-5 × 10⁷ infectious particles) were quantified by Taqman qPCR assay as described elsewhere (43).

**Lentiviral transduction.** The RINm5F-control, RINm5F-Cat, RINm5F-MitoCat (33), INS-1E, and primary rat islet cells were infected with HyPer-Mito or HyPer-Peroxi lentivirus at a MOI of 10. The tissue culture cells were selected for HyPer expression using blasticidin (1 µmol/L).

**Analysis of H₂O₂ generation using HyPer proteins.** RINm5F cells or primary rat islet cells that overexpressed HyPer-Mito or HyPer-Peroxi were seeded onto black 24-well glass-bottom plates (Greiner, Frickenhausen, Germany). Cells were cultured for 24 h and afterwards exposed to palmitic acid for another 24 h. Live cell imaging was performed using a CFP-YFP dual filter (excitation, 427 nm and 504 nm; emission, 520 nm) with a cellR/Olympus IX 81 inverted microscope system and CellR software (Olympus, Hamburg, Germany) for imaging and analysis.

To determine H₂O₂ production, changes in
the fluorescence ratios of RINm5F, RINm5F-Cat, RINm5F-MitoCat, and INS-1E cells that overexpressed HyPer-Mito or HyPer-Peroxi were quantified spectrofluorometrically. Those cells were seeded at 25,000 cells per well onto black 96-well plates and cultured for 24 h. The fluorescence ratio was measured immediately before and after 24 h treatment with palmitic acid.

**Immunocytochemical staining.** For immunocytochemical staining of primary rat β-cells, HepG2 cells, RINm5F cells, and RINm5F cells that overexpressed catalase, 100,000 cells were seeded overnight on collagen-coated glass slides and subsequently fixed with 4% paraformaldehyde. After washing, the cells were permeabilized and blocked with PBS containing 0.2% Triton X-100 and 1% BSA. The slides were incubated with primary antibodies (for detailed information see Supplementary Table S1 in the online appendix at [http://diabetes.diabetesjournals.org](http://diabetes.diabetesjournals.org)) diluted in PBS containing 0.1% Triton X-100 and 0.1% BSA at room temperature for 60 min. Then, the cells were washed with PBS and incubated with secondary antibodies (for detailed information see Supplementary Table S1) for 60 min. For nuclear counterstaining, 300 nM DAPI was applied for 5 min at room temperature. Finally, the cells were washed and mounted with Mowiol/DABCO (Sigma, St. Louis, MO, USA) anti-photobleaching mounting media. Stained cells were examined on an Olympus IX81 inverted microscope (Olympus, Hamburg, Germany) and microscopic images were post-processed using AutoDeblur and AutoVisualize (Autoquant Imaging, New York, USA).

**Statistical analysis.** Data are expressed as means ± SEM. Statistical analyses were performed using ANOVA plus Dunnett’s test for multiple comparisons, unless stated otherwise. EC$_{50}$ values were calculated from nonlinear regression analyses using least square algorithms of the Prism analysis program (Graphpad, San Diego, CA, USA).

**RESULTS**

**Chain length-dependent toxicity of saturated non-esterified fatty acids in insulin-producing cells.** Saturated NEFAs were toxic to RINm5F insulin-producing cells. In both the MTT cell viability and the propidium iodide (PI) cytotoxicity assays, toxicity was dependent on NEFA chain length (Fig. 1). The longest chain length fatty acid analyzed, stearic acid (C18:0), was the most toxic, with a half-maximal effective concentration (EC$_{50}$) below 100 µM. The EC$_{50}$ for palmitic acid (C16:0), the physiologically most abundant and important saturated NEFA, was around 100 µM after 24 h incubation. Toxicity decreased with decreasing chain length. Myristic acid (C14:0) was highly toxic (EC$_{50}$ ≅ 200 µM), while tridecanoic acid (C13:0) was significantly less toxic, with an EC$_{50}$ well above 500 µM. With further chain length shortening (C12:0 to C10:0), toxicity was negligible with EC$_{50}$ values exceeding 1000 µM. For even shorter chain saturated fatty acids (C8:0 to C4:0), EC$_{50}$ values could not be calculated due to marginal toxicity (data not shown). Toxicity was also observed in a FACS-based caspase-3 assay (30% caspase-3 positive cells at 100 µM palmitic acid as compared to 6% caspase-3 positive cells under control conditions), indicating an apoptotic mode of fatty acid-induced cell death.

Palmitic acid-induced cell death was also observed in isolated primary rat islet cells in the propidium iodide cytotoxicity assay. After 24 h incubation with 500 µM palmitic acid 33% ± 4 of rat islet cells stained positive for propidium iodide, whereas in untreated cells the cell death rate was low (6% ± 1).
While primary rat islet cells as well as untransfected RINm5F insulin-producing cells showed virtually no immunostaining for catalase (data not shown), RINm5F insulin-producing cells transfected to overexpress catalase in the cytosol (Cat) showed a homogenous distribution of catalase in the cytoplasm and a dot-like pattern of catalase co-localization with peroxisomes (Fig. 2A); on the other hand, catalase did not co-localize with mitochondria (Fig. 2B). Catalase was not detectable in the cytoplasm or in the peroxisomes of insulin-producing cells that overexpressed catalase in the mitochondria (Mito-Cat; Fig. 2C); in these cells, catalase co-localized specifically with the mitochondria (Fig. 2D).

The localization of catalase in RINm5F-Cat cells was additionally analysed in 3D images using the Imaris software (Bitplane, Zurich, Switzerland). The 3D models were generated from 20 z-layers of the immunocytochemical staining of catalase and the peroxisomes (Supplementary Fig. S1) or the mitochondria (Supplementary Fig. S2). In these images, catalase showed a clear co-localization with the peroxisomes while no significant co-localisation was detectable with the mitochondria.

The results of these immunostainings were also reflected by the catalase enzyme activities of the examined cells. Primary rat islet cells and untransfected RINm5F cells showed a comparable catalase activity of 20 ± 5 U/mg and 13 ± 1 U/mg, respectively. By contrast in RINm5F-catalase (353 ± 7 U/mg) and RINm5F-mito-catalase (367 ± 43 U/mg) cells the enzyme activity was approximately 17-fold higher.

**Effects of the antioxidative enzymes catalase and SOD on palmitic acid toxicity in insulin-producing cells.** RINm5F insulin-producing cells that overexpressed different antioxidative enzymes were used as a tool to analyze the role of ROS in palmitic acid toxicity. While overexpression of the H₂O₂-inactivating enzyme catalase in peroxisomes and in the cytosol (Cat) protected against palmitic acid (100 µM) toxicity, catalase overexpression in the mitochondria (Mito-Cat) did not provide protection (Fig. 3).

Neither overexpression of the cytosolic superoxide radical detoxifying isoenzyme CuZnSOD (copper zinc superoxide dismutase) nor overexpression of the mitochondrial isoenzyme MnSOD (manganese superoxide dismutase) provided significant protection against palmitic acid toxicity.

**Overexpression of catalase in insulin-producing cells reduced palmitic acid-induced ROS generation.** ROS generation in RINm5F insulin-producing control cells as measured by the DCF fluorescence method was increased by about 40% in response to exposure to 100 µM palmitic acid (Fig. 4). Overexpression of catalase in the peroxisomes and the cytosol, but not in the mitochondria, significantly reduced palmitic acid-induced (100 µM) ROS generation.

**Peroxisomes in rat islet cells, RINm5F insulin-producing cells, and HepG2 cells.** Peroxisomes were clearly detectable by immunofluorescence as small green spots in primary rat β-cells, RINm5F insulin-producing cells, and HepG2 cells (Supplementary Fig. S3). β-cells isolated from rat pancreatic islets were identified by immunofluorescent staining of insulin; RINm5F cells were also positive for insulin. To quantify the density of peroxisomes in insulin-producing and hepatoma cells the peroxisomes were counted in β-cells, RINm5F cells, and HepG2 cells after immunofluorescence staining of the peroxisomes by PMP-70 in 5-8 images (Supplementary Fig. S4). The number of peroxisomes per cell was determined by the image analysis software Imaris (Bitplane, Zurich, Switzerland). The results clearly show that there were no significant differences in peroxisome density in hepatoma and in
insulin-producing RINm5F cells as well as in primary rat β-cells.

**Intracellular localization of the HyPer-Peroxi protein.** To prove the intracellular localization of the HyPer protein targeted to the peroxisomes in RINm5F cells, fixed cells were stained for the peroxisomal membrane protein 70 (PMP-70). The fluorescence (504/520 nm) of the HyPer protein was clearly detectable as green spots after fixation with paraformaldehyde. Staining of the peroxisomes (PMP-70) showed a distinct co-localization with the HyPer-Peroxi protein (Supplementary Fig. S5 A-C).

**Subcellular site of H$_2$O$_2$ generation in insulin-producing cells following exposure to palmitic acid.** In order to obtain more information about the nature of the reactive oxygen species and the intracellular site of ROS production following palmitic acid exposure, the HyPer protein, which is a specific sensor for H$_2$O$_2$, was expressed in either the peroxisomes (RINm5F-HyPer-Peroxi) or the mitochondria (RINm5F-HyPer-Mito) of RINm5F insulin-producing cells. The HyPer protein allows ratiometric measurement of H$_2$O$_2$ by fluorescence microscopy at two different wavelengths. In the false-colored overlay images, green staining corresponds to low, yellow to medium, and red to high H$_2$O$_2$ concentrations. After 24 h of exposure to palmitic acid, RINm5F-HyPer-Peroxi cells showed a strong shift towards red fluorescence (Fig. 5C and 5D). The quantification revealed a significant 240% increase of H$_2$O$_2$ production after 24 h palmitic acid treatment (Fig.5E). In contrast, the RINm5F-HyPer-Mito cells showed only a slight non-significant increase in red fluorescence, indicating a lower H$_2$O$_2$ concentration in the mitochondria relative to the peroxisomes (Fig. 5A, 5B and 5E). RINm5F-HyPer-Peroxi and RINm5F-HyPer-Mito cells incubated without palmitic acid under control conditions for 24 h showed no changes in fluorescence signals (data not shown).

The key finding of H$_2$O$_2$ generation in the peroxisomes of RINm5F cells after exposure to palmitic acid was confirmed in primary rat islet cells. Single rat islet cells were lentivirally transduced with Hyper-Peroxi cDNA and incubated with palmitic acid for 24 h. In the palmitic acid treated islet cells a clear shift towards red fluorescence was detectable, which corresponds to a higher H$_2$O$_2$ concentration in comparison to untreated cells (Fig. 6). The fluorescence shift of single cells was quantified with the CellIR software (Olympus, Hamburg, Germany). Palmitic acid treatment induced a significant 125% increase of H$_2$O$_2$ production in comparison to the control condition (Fig. 6E).

**Quantification of H$_2$O$_2$ generation in insulin-producing cells following exposure to palmitic acid.** Differences in peroxisomal and mitochondrial H$_2$O$_2$ concentration following 24 h exposure to palmitic acid were quantified spectrofluorometrically. Treatment with 100 µM palmitic acid induced a significantly higher rate of H$_2$O$_2$ production in both the peroxisomes and the mitochondria of control RINm5F cells than did treatment with 50 µM palmitic acid (Fig. 7A and B). At 100 µM palmitic acid, there was a tendency for a greater increase of the peroxisomal as compared to the mitochondrial H$_2$O$_2$ production in RINm5F cells (Fig. 7A and B). In INS-1E cells the H$_2$O$_2$ production was even significantly higher in the peroxisomes than in the mitochondria after palmitic acid treatment (Fig. 8).

Additional information about the intracellular site of H$_2$O$_2$ production after palmitic acid exposure was obtained by overexpressing the H$_2$O$_2$ detoxifying enzyme catalase either in the peroxisomes and the cytosol or in the mitochondria of RINm5F-HyPer-Peroxi and RINm5F-HyPer-Mito cells, respectively. Catalase expression in the peroxisomes and cytosol of RINm5F-HyPer-
Hydrogen peroxide mediated lipotoxicity

Peroxi cells reduced H$_2$O$_2$ production significantly in the presence of 50 and 100 µM palmitic acid as compared to control cells, while overexpression of catalase in the mitochondria did not significantly reduce H$_2$O$_2$ production in the peroxisomes (Fig. 7A).

Overexpression of catalase in the peroxisomes and the cytosol significantly reduced the mitochondrial H$_2$O$_2$ concentration in response to incubation with 100 µM (and to a lesser extent with 50 µM) palmitic acid as compared to control cells. Overexpression of catalase in the mitochondria did not significantly affect mitochondrial production of H$_2$O$_2$ even though there was a tendency towards a reduction at 100 µM palmitic acid (Fig. 7B).

Overall, the data showed that overexpression of catalase in peroxisomes and cytosol reduced H$_2$O$_2$ generation in the peroxisomes and, to a lesser extent, in the mitochondria. In contrast, overexpression of catalase in the mitochondria had no significant effect on H$_2$O$_2$ production in either organelle (compare Fig. 7A and 7B).

DISCUSSION

The present data clearly show that NEFA toxicity increases with increasing chain length. NEFAs are oxidized not only in the mitochondria, but also in the peroxisomes. Fatty acid β-oxidation is a property of peroxisomes in most, if not all, organisms (27). Moreover, in yeast and plants, peroxisomes are the sole sites of fatty acid β-oxidation (44). One product of peroxisomal β-oxidation is H$_2$O$_2$, whereas mitochondrial β-oxidation generates reducing equivalents (27; 28). Mitochondrial β-oxidation is tightly coupled to the respiratory chain and oxidative phosphorylation. It provides acetyl-CoA for further oxidation in the TCA cycle and ensures the production of ATP, which is not an apparent function of peroxisomal β-oxidation. In peroxisomal β-oxidation, the initial step generates H$_2$O$_2$ and energy is lost as heat. Peroxisomal β-oxidation results in chain shortening of long- and very long-chain fatty acids, which are poor substrates for mitochondrial β-oxidation. The shortened fatty acids are subsequently transported to the mitochondria in a carnitine-dependent manner for further degradation (27; 28).

The observation of the chain length-dependent increase in the toxicity of saturated fatty acids to insulin-producing cells prompted us to study the role of oxygen free radicals generated by peroxisomal β-oxidation as mediators of lipotoxicity. In the first step of peroxisomal β-oxidation, FAD-containing acyl-CoA oxidase introduces a double bond at the β-position of the fatty-acyl-CoA ester and the hydrogen atoms are transferred to molecular oxygen to yield H$_2$O$_2$ (28). Remarkably, in rat liver for example about 20% of total oxygen consumption is accounted for peroxisomal oxidase activity (45). For the detoxification of H$_2$O$_2$, the oxidoreductase catalase, which has a high turnover rate, is expressed in the peroxisomes of most tissues (28) but not in those of pancreatic β-cells (29; 30). This low catalase enzyme activity could also be found in RINm5F cells, which makes these cells well-suited as model cells for H$_2$O$_2$ mediated lipotoxicity.

Experiments with insulin-producing cells overexpressing antioxidative enzymes showed that only cytosolic catalase provided protection against palmitic acid-induced toxicity; mitochondrial catalase was not protective. The superoxide radical detoxifying isoenzymes MnSOD and CuZnSOD were also not protective, indicating that the formation of superoxide radicals does not play as crucial a role in lipotoxicity as does the formation of H$_2$O$_2$. Indeed, determination of ROS production by DCF fluorescence measurements provided support for this hypothesis by showing a reduction in ROS generation in insulin-producing cells that
overexpressed catalase in the peroxisomes and the cytosol but not in the mitochondria. Using the H$_2$O$_2$-sensitive HyPer protein as a novel, specific method for detecting H$_2$O$_2$, we clearly identified H$_2$O$_2$ as the main reactive oxygen species formed during palmitic acid treatment. To determine the subcellular site of H$_2$O$_2$ formation, the HyPer protein was fused to a peroxisome- or a mitochondrion-targeting sequence to allow organelle-specific expression.

These experiments show that peroxisomes were a major site of H$_2$O$_2$ formation in insulin-producing cells, while the mitochondria were a minor site. Primary rat islet cells are well equipped with peroxisomes, and showed also an increased peroxisomal H$_2$O$_2$ formation in response to palmitic acid, as demonstrated in this study.

An additional argument for this hypothesis is the fact that overexpression of catalase in the peroxisomes and the cytosol (RINm5F-Cat) significantly reduced H$_2$O$_2$ production not only in the peroxisomes but also in the mitochondria, though catalase was not overexpressed in the latter organelle. H$_2$O$_2$ as a membrane-permeable ROS (23; 24) can diffuse from its site of generation in the peroxisome into the mitochondria where it is detected through the HyPer-Mito protein. Thus the source of the elevated H$_2$O$_2$ concentration detected in the mitochondria is at least in part H$_2$O$_2$ generated in the peroxisomes.

However, in contrast to the peroxisomes in other cell types, insulin-producing cells do not appear to express catalase mRNA or protein (29; 30). This lack of catalase expression leaves insulin-producing cells badly protected against potentially hazardous effects of H$_2$O$_2$ generated through peroxisomal β-oxidation. Mitochondrial β-oxidation may not be able to cope with the elevated levels of NEFAs that are associated with obesity and type 2 diabetes, resulting in a higher proportion of fatty acids being metabolized through peroxisomal β-oxidation, leading to increased H$_2$O$_2$ formation. The membrane-permeable H$_2$O$_2$, could leave the peroxisomes and harm insulin biosynthesis and secretion (11; 46; 47), as has been shown for insulin-secreting cells after exposure to high NEFA concentrations for extended periods (15). The results of the present study indicate that ROS generated in the peroxisomes are the major cause of lipotoxicity-mediated β-cell dysfunction. This does not detract from the fact that mitochondrial ROS formation may contribute to this phenomenon in particular with respect to direct negative effects on mitochondrial function.

NADPH oxidase activation could also be a source of long-chain fatty acid-mediated superoxide radical generation (22) that has been postulated to cause lipotoxicity in insulin-producing cells (15; 48). Palmitic acid has been shown to activate this plasma membrane enzyme in aortic smooth muscle and endothelial cells in a protein kinase C-dependent manner, leading to superoxide radical formation (49). In insulin-producing cells, palmitic acid can induce superoxide radical formation along with increased expression of the NADPH oxidase p47$^{phox}$ subunit (15; 48; 50). However, data from the present study indicated that cytosolic CuZnSOD did not protect insulin-producing cells against palmitic acid-induced toxicity, suggesting that an increased rate of superoxide radical formation per se is insufficient to explain NEFA-induced lipotoxicity.

Thus, we hypothesize that NEFA-induced lipotoxicity is mediated by H$_2$O$_2$ generated during peroxisomal β-oxidation of palmitic acid as the physiologically most abundant long-chain saturated fatty acid. This provides an interesting new concept for lipid-induced glucose intolerance in obesity and diabetic hyperglycemia as a result of β-cell dysfunction in patients with type 2 diabetes.
The lack of catalase expression in pancreatic β-cell peroxisomes (29; 30) explains the exceptional susceptibility of pancreatic β-cells to lipotoxicity (14; 15; 23).

Author Contributions. M.E. wrote manuscript, researched data. W.G. wrote manuscript, researched data. S.L. wrote manuscript.

ACKNOWLEDGEMENTS
We are grateful to Maren Böger for her skillful technical assistance. This work was supported by the European Union (Integrated Project EuroDia LSHM-CT-2006-518153 in the Framework Program 6 [FP6] of the European Community).

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Fig. 1 Toxicity of saturated non-esterified fatty acids (NEFAs) according to chain length (C10:0-C18:0) in RINm5F insulin-producing cells
Cells were incubated for 24 h with saturated NEFAs of different chain lengths and viability was determined by MTT assay (A) or propidium iodide staining (B). EC50 values were calculated by nonlinear regression analysis. Data are means ± SEM from 4-6 individual experiments. *p < 0.01 as compared to C10:0, C11:0, C12:0, or C13:0. Other comparisons were not significant (ANOVA/Tukey’s test for multiple comparisons).
Fig. 2 Immunocytochemical staining for catalase, peroxisomes, and mitochondria in catalase or mitocatalase overexpressing RINm5F insulin-producing cells
RINm5F insulin-producing cells that overexpressed catalase in the cytosol (Catalase, A and B) or in the mitochondria (Mito-Catalase, C and D) were seeded overnight on collagen-coated coverslips. After fixation with 4% paraformaldehyde, the cells were stained for catalase (red) and for the peroxisomal membrane protein 70 (PMP-70 green) or the mitochondrial respiratory chain enzyme cytochrome c-oxidase IV (COX-4 green) followed by nuclear counterstaining with DAPI (blue). To quantify the colocalization between catalase and the peroxisomes or mitochondria 20 images of 2 independent preparations were analyzed with the Colocalization Add-In of the CellR software (Olympus, Hamburg, Germany). The analyses showed that 56.2% ± 3.3 (n=58) of catalase were localized in the peroxisomes (A) and 5% ± 0.4 (n=74) in the mitochondria (B). For the Mito-Catalase expressing cells a proportion of 86.3% ± 2.6 (n=90) of catalase was detected in the mitochondria (C) and 5.1% ± 1.6 (n=62) in the peroxisomes (D). Data are means ± SEM of (n) individual cells.
Fig. 3 Palmitic acid toxicity in RINm5F insulin-producing cells that overexpress different antioxidative enzymes
RINm5F cells that stably overexpressed the cytosolic antioxidative enzymes copper zinc superoxide dismutase (CuZnSOD) and catalase (Cat) or the mitochondrial antioxidative enzymes manganese superoxide dismutase (MnSOD) and catalase with a mitochondrial leader sequence (Mito-Cat) were incubated with palmitic acid (100 µM) for 24 h; viability was determined by MTT assay. Mock-transfected RINm5F cells served as controls; untreated cells were set as 100% viability. Data are means ± SEM from five individual experiments **p < 0.01 as compared to control cells (ANOVA/Dunnett’s test for multiple comparisons).

Fig. 4 Palmitic acid-induces production of reactive oxygen species in RINm5F insulin-producing cells that overexpress catalase in the cytosol (Cat) or in the mitochondria (Mito-Cat)
To determine ROS generation, cells were loaded with 10 µM of DCF-DA dye for 30 min and then cultured with 100 µM palmitic acid (PA) for 24 h. DCF fluorescence was measured after 24 h and normalized to that of untreated cells. Data are means ± SEM from seven individual experiments. ##p < 0.01 as compared with untreated cells (t-test, unpaired, two-tailed); **p < 0.01 as compared with control cells (ANOVA/Dunnett’s test for multiple comparisons).
Fig. 5 Live cell fluorescence microscopy for detection of H$_2$O$_2$ in RINm5F insulin-producing cells using the H$_2$O$_2$ sensor proteins HyPer-Mito and HyPer-Peroxi

Cells that stably expressed the H$_2$O$_2$ sensor protein HyPer in the mitochondria (HyPer-Mito A and B) or peroxisomes (HyPer-Peroxi C and D) were incubated with 100 µM palmitic acid for 24 h. Shown are representative images at 0 h (A and C) and 24 h (B and D). Fluorescence at 504/520 nm is depicted in red and fluorescence at 427/520 nm is shown in green. Increased H$_2$O$_2$
Hydrogen peroxide mediated lipotoxicity generation is indicated by a color change from green to yellow to red. The group specified as “PA 0 h” comprises cells which were analyzed immediately after NEFA treatment (0 h). (E) To quantify the hydrogen peroxide production images of RINm5F cells were analyzed with the CellR software (Olympus, Hamburg, Germany). The fluorescence intensities of individual cells were measured at 504/520 nm and 427/520 nm and the ratio of both wavelength pairs indicates the H$_2$O$_2$ production. Data are means ± SEM from four individual experiments. *p < 0.05 as compared with H$_2$O$_2$ production at 0 h (t-test, unpaired, two-tailed).

Fig. 6 Live cell fluorescence microscopy for detection of H$_2$O$_2$ in primary rat islet cells using the H$_2$O$_2$ sensor protein HyPer-Peroxi
Primary rat islet cells were infected with HyPer-Peroxi lentivirus at a MOI of 10. Islet cells
which expressed the H$_2$O$_2$ sensor protein HyPer in peroxisomes (HyPer-Peroxi) were incubated with 500 µM palmitic acid (PA) for 24 h (C and D). Shown are representative images at 0 h (A and C) and 24 h (B and D). Fluorescence at 504/520 nm is depicted in red and fluorescence at 427/520 nm is shown in green. Increased H$_2$O$_2$ generation is indicated by a color change from green to yellow to red. The group specified as “PA 0 h” comprises cells which were analyzed immediately after NEFA treatment (0 h). Cells specified as “untreated” were cultivated under control conditions in medium with 1% ethanol and the appropriate BSA concentration (BSA:NEFA ratio of 2% : 1 mM) in the absence of NEFAs. (E) To quantify the hydrogen peroxide production images of primary rat islet cells were analyzed with the CellR software (Olympus, Hamburg, Germany). The fluorescence intensities of individual cells was measured at 504/520 nm and 427/520 nm and the ratio of both wavelength pairs indicates the H$_2$O$_2$ production. Data are means ± SEM from four individual experiments. *p < 0.05 as compared with H$_2$O$_2$ production at 0 h (t-test, unpaired, two-tailed).

Fig. 7 Localization of H$_2$O$_2$ production in RINm5F insulin-producing cells after palmitic acid treatment
Cells that stably expressed the H$_2$O$_2$ sensor protein HyPer in peroxisomes (A) or mitochondria (B) and catalase in the cytosol (Cat) or mitochondria (Mito-Cat) were treated with 50 or 100 µM palmitic acid (PA) for 24 h. The fluorescence ratio (504/520 nm to 427/520 nm), which is an indicator of H$_2$O$_2$ production, was measured spectrofluorometrically. Shown are the changes in the fluorescence ratios after 24 h normalized to the fluorescence ratios of untreated cells. Data are means ± SEM from 10 individual experiments. #p < 0.05, ##p < 0.01 vs. untreated cells (0 µM PA), *p < 0.05, **p < 0.01 vs. control cells (ANOVA/Dunnett’s test for multiple comparisons).

**Fig. 8 Localization of H$_2$O$_2$ production in INS-1E insulin-producing cells after palmitic acid treatment**

Cells that stably expressed the H$_2$O$_2$ sensor protein HyPer in the peroxisomes or the mitochondria were treated with 500 µM palmitic acid for 24 h. The fluorescence ratio (504/520 nm to 427/520 nm), which is an indicator of H$_2$O$_2$ production, was measured spectrofluorometrically. Shown are the changes in the fluorescence ratios after 24 h normalized to the fluorescence ratios of untreated cells. Data are means ± SEM from 14 individual experiments. *p < 0.05 vs. HyPer-Peroxi; ## < 0.01 vs. untreated cells (t-test, unpaired, two-tailed).