Role of metabolically generated reactive oxygen species for lipotoxicity in pancreatic β-cells

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Chronically elevated concentrations of non-esterified fatty acids (NEFAs) in type 2 diabetes may be involved in β-cell dysfunction and apoptosis. It has been shown that long-chain saturated NEFAs exhibit a strong cytotoxic effect upon insulin-producing cells, while short-chain as well as unsaturated NEFAs are well tolerated. Moreover, long-chain unsaturated NEFAs counteract the toxicity of palmitic acid. Reactive oxygen species (ROS) formation and gene expression analyses together with viability assays in different β-cell lines showed that the G-protein-coupled receptors 40 and 120 do not mediate lipotoxicity. This is independent from the role, which these receptors, specifically GPR40, play in the potentiation of glucose-induced insulin secretion by saturated and unsaturated long-chain NEFAs. Long-chain NEFAs are not only metabolized in the mitochondria but also in peroxisomes. In contrast to mitochondrial β-oxidation, the acyl-coenzyme A (CoA) oxidases in the peroxisomes form hydrogen peroxide and not reducing equivalents. As β-cells almost completely lack catalase, they are exceptionally vulnerable to hydrogen peroxide generated in peroxisomes. ROS generation in the respiratory chain is less important because overexpression of catalase and superoxide dismutase in the mitochondria do not provide protection. Thus, peroxisomally generated hydrogen peroxide is the likely ROS that causes pancreatic β-cell dysfunction and ultimately β-cell death.

Keywords: β-oxidation, G-protein-coupled receptors, insulin secretion, mitochondria, non-esterified fatty acids, peroxisomes, reactive oxygen species, type 2 diabetes mellitus

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Introduction

Type 2 diabetes mellitus is a complex metabolic disorder with a dramatically increasing prevalence worldwide [1]. This disorder is characterized by peripheral insulin resistance and pancreatic β-cell dysfunction [2,3], resulting in defective glucose-induced insulin secretion [4–6] and ultimately in β-cell loss through apoptosis [7,8]. Hypercaloric Western diets, rich in carbohydrates and saturated fats, are responsible for the manifestation of the metabolic syndrome. This is characterized by dyslipidaemia, hypertension, and obesity, which precede type 2 diabetes manifestation. Accompanying elevated levels of non-esterified fatty acids (NEFAs) [9] can suppress insulin secretion and cause β-cell dysfunction and loss, a phenomenon referred to as lipotoxicity [10,11]. Although lipotoxicity is subject to intensive research and scientific discussion, a conclusive molecular mechanism has not been elucidated.

Structural Requirements for Lipotoxicity

The effects of NEFAs upon insulin-producing cells are dependent on chain length and degree of saturation [12]. Saturated long-chain NEFAs, such as the physiologically most abundant saturated NEFA palmitic acid, are highly toxic, whereas NEFAs with a shorter chain length (C13:0 tridecanoic acid or shorter) are well tolerated by insulin-producing cells (Elsner et al., in prep.). By contrast, unsaturated NEFAs, irrespective of their chain length, are not toxic. Moreover, unsaturated long-chain NEFAs, like the physiologically most important oleic acid, attenuate the toxic effect of saturated long-chain NEFAs such as palmitic acid. This protective potency decreases with shortening of the chain length (Elsner et al., in prep.) [12]. Monounsaturated NEFAs with a chain length of C12 or C14 are clearly less protective against palmitic acid-induced toxicity than fatty acids with a chain length of ≥C16. Furthermore, the protective potency of long-chain unsaturated NEFAs is not dependent on the number of double bonds; thus, oleic (C18:1), linoleic (C18:2) and γ-linolenic acid (C18:3) show a comparable protective effect (Elsner et al., in prep.). Therefore, the different NEFAs can be subdivided into three different groups, depending upon their effect on β-cell viability: (i) saturated long-chain NEFAs, which are toxic; (ii) unsaturated long-chain NEFAs, which attenuate the cytotoxic effect of saturated long-chain NEFAs and (iii) saturated and unsaturated short-chain NEFAs, which are neither toxic nor protective. These different effects of NEFAs in dependence on their structure are indicative of an involvement of a receptor-mediated signalling cascade. Within the family of G-protein-coupled receptors (GPRs), some are known to bind specifically fatty acids, such as GPR40, GPR41, GPR43, GPR119 and GPR120.
Fatty Acid Binding Receptors

The family of GPRs shares the structural feature of seven transmembrane α-helices. These receptors translate a signal into an intracellular response through heterotrimeric G proteins. These GPRs are expressed in almost all tissues and are activated by a broad variety of ligands, like hormones, neurotransmitters, peptides, proteins, steroids as well as NEFAs. The GPR isoforms 40, 41, 43, 119 and 120 belong to the subset of lipid-binding receptors [13,14]. All isoforms have been considered to be expressed in insulin-secreting β-cells, although information about the extent of expression is inconsistent [15]. GPR119 and GPR120 both belong to the rhodopsin (class A) family, which showed only slight homology to GPR40, 41 or 43 [14]. GPR119 is mainly expressed in the pancreas and the gastrointestinal tract [16]. However, the fact that this receptor is activated only by fatty acid derivatives, such as lysophosphatidylcholine (LPC) and oleoyl ethanolamide (OEA) [16,17], excludes GPR119 as a mediator for lipotoxicity of saturated long-chain NEFAs.

The GPR120 is a potentially interesting candidate, as it is specifically activated through long-chain NEFAs [18]. Analysis of tissue distribution showed the highest expression levels in the intestinal tract, although it has also been found in different β-cell lines (figure 1) [12,15]. Activation of this receptor isoform by unsaturated long-chain NEFAs, such as α-linolenic acid, results in glucagon-like peptide-1 (GLP-1) secretion from the enteroendocrine cell line STC-1, through activation of ERK (extracellular-signal regulated kinase) and PI3 kinase (phosphatidylinositol 3-kinase) pathways [18]. Interestingly, the lack of protection against saturated long-chain NEFAs (Elsner et al., in prep.) [22] argue against an involvement of these receptor isoforms in lipotoxicity or cytoprotection of unsaturated NEFAs. GPR40 has been considered to be the most promising candidate among these receptors as a lipotoxicity mediator. It is abundantly expressed in human and rodent islets [23,24] and could also be found in several β-cell lines (figure 1) [24]. Saturated and unsaturated medium to long-chain NEFAs (C12-C18) are the preferred ligands of GPR40.

Fatty Acids and Insulin Secretion

Interestingly, these NEFAs also potentiate glucose-induced insulin secretion from pancreatic β-cells through activation of GPR40 (figure 2) [24]. Binding of the fatty acids to GPR40 activates the Goq-phospholipase C (PLC) pathway resulting in an increase in cytosolic-free [Ca2+]i through inositol trisphosphate (IP3)-mediated release of Ca2+ from endoplasmic reticulum (ER) stores [25,26]. In addition, signalling via PLC and diacylglycerol (DAG) may contribute to exocytosis of insulin (figure 3). A contribution of Ca2+ influx through L-type Ca2+ channels has also been considered [25,26]. Thus, emptying of intracellular Ca2+ stores in β-cells can induce a depolarizing current that increases Ca2+ influx through voltage-dependent

![Figure 1](image1.png)  
**Figure 1.** Gene expression analyses of GPR40 and GPR120 in different insulin-producing cell lines. The mRNA expression of GPR40 (black bars) and GPR120 (open bars) in RINm5F, INS-1E and MIN6 cells was quantified by qPCR. Expression levels were normalized to the expression of the reference gene actin. Results are means ± s.e.m. from three to five individual experiments.

![Figure 2](image2.png)  
**Figure 2.** Non-esterified fatty acid (NEFA)-mediated enhancement of glucose-induced insulin secretion. Insulin secretion from MIN6 and INS-1E cells in the presence of different glucose concentrations after a 2 h incubation with 150 μM of palmitic acid (PA), oleic acid (OA) or a mixture of both (150 μM PA + 150 μM OA). Results are means ± s.e.m. from four to six individual experiments; *p < 0.05, **p < 0.01, ***p < 0.001 compared with untreated cells (ANOVA/Dunnett’s test).
The short-term effects of non-esterified fatty acids (NEFAs) in comparison with the long-term effects in the pancreatic \( \beta \)-cell. NEFAs exert two different effects on \( \beta \)-cells: the short-term effect is an enhancement of glucose-induced insulin secretion via GPR40 and the long-term effect is the induction of lipotoxicity via an enhancement of peroxisomal metabolism, yielding high levels of \( \text{H}_2\text{O}_2 \) ultimately leading to apoptosis.

Ca\(^{2+}\) channels [27]. The insulinotropic effect of unsaturated fatty acids, such as oleic acid, is somewhat weaker than saturated fatty acids, such as palmitic acid (figure 2) [28]. A combination of palmitic acid and oleic acid causes a similar strong insulin-secretory response to palmitic acid alone (figure 2).

The GPR isoforms, such as GPR41 and GPR43, as well as GPR119 have a fatty acid binding profile that cannot explain the strong potentiating effect of long-chain saturated and unsaturated NEFAs, although it has been shown that LPC enhances glucose-stimulated insulin secretion via GPR119-mediated stimulation of adenylate cyclase and intracellular cyclic adenosine monophosphate (cAMP) accumulation [16]. Only the GPR40 and GPR120 receptors, with their characteristic features, classify these fatty acid plasma membrane receptors as crucial mediators of palmitic and oleic acid-mediated potentiation of glucose-induced insulin secretion. And in fact, NEFA-mediated potentiating effects are reduced in parallel with a reduction of GPR40 expression [24,29]. The potential role of GPR120 for enhanced insulin secretion, on the other hand, requires further investigation.

Long-chain acyl CoA (LC-CoA) esters, which have been considered for some time to play a crucial signalling role in fatty acid-mediated potentiation of insulin secretion [30,31], are not central for the effects of NEFAs on insulin secretion. The metabolism of fatty acids is apparently of subordinate importance as a site of generation of a specific metabolic signal for fatty acid-mediated insulin secretion. It may be possible, however, that metabolites generated within mitochondrial \( \beta \)-oxidation and subsequent citric acid cycle, as assumed for the potentiation of glucose-induced insulin secretion through the amplifying pathway [32–35], contribute in a collaborative manner to the insulin-secretory capacity of fatty acids.

In a sense the mechanism of fatty acid potentiation resembles the mechanisms of other potentiators of glucose-induced insulin secretion such as acetylcholine, bombesin or arginine vasopressin, which mediate their effects via interaction with a receptor in the \( \beta \)-cell plasma membrane coupled to signalling pathways that increase intracellular \( \text{Ca}^{2+} \), through mobilization from the ER and increased \( \text{Ca}^{2+} \) influx through L-type \( \text{Ca}^{2+} \) channels [25]. In particular, in the phase of insulin resistance before diabetes manifestation, when an increased \( \beta \)-cell
volume compensates for higher insulin demands of the obese organism, the potentiation of glucose-induced insulin secretion by elevated fatty acid concentrations in the circulation is operative.

GPR40 Receptor and Lipotoxicity-mediated β-Cell Dysfunction

The GPR40 receptor has also been considered to mediate lipotoxicity in insulin-secreting cells [36]. However, a number of experimental observations have been made, which are not compatible with a central role of the GPR40 receptor in lipotoxicity. (i) The substrate specificity of the GPR40 receptor is not congruent with the pattern of NEFA toxicity. (ii) Lipotoxicity is not dependent upon the expression of the GPR40 receptor in insulin-secreting cells. (iii) Pancreatic β-cells are damaged also in GPR40 knockout (KO) mice fed a high-fat diet. (iv) Small molecule agonists (e.g. Cpd-B) of GPR40 potentiate glucose-induced insulin secretion in the same way as NEFAs, but are not toxic and do not impair insulin secretion.

In detail, (i) saturated and unsaturated NEFAs exert completely different effects on β-cell viability. Thus, long-chain saturated NEFAs showed a strong cytotoxic effect, whereas unsaturated long-chain NEFAs are well tolerated (Elsner et al., in prep.) [12,37]. Binding to GPR40 cannot explain these different effects, because both saturated and unsaturated long-chain NEFAs are ligands for GPR40. Another important point is the time course of these effects; because both saturated and unsaturated long-chain NEFAs are ligands for GPR40. Another important point is the time course of these effects; enhanced insulin secretion via GPR40 is evident within hours after exposure, whereas toxicity is only obvious after 1–3 days.

(ii) GPR40 expression is different in the analysed β-cell lines. The highest expression level was found in MIN6 cells, in INS-1E cells the expression was somewhat lower and...
completely absent in RINm5F cells (figure 1). Interestingly, these cell lines also showed a different sensitivity for palmitic acid-induced toxicity in an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) viability assay after 24 h treatment. RINm5F cells were by far the most sensitive cells with an EC50 (half maximally effective concentration) value for palmitic acid of 98 μM ± 5. For MIN6 and INS-1E cells, the EC50 values of 993 μM ± 123 and 1860 μM ± 540, respectively, were significantly higher. Therefore, it is unlikely that GPR40 mediates palmitic acid-induced toxicity, because the receptor is not expressed in the most sensitive RINm5F cells.

(iii) Studies with GPR40 KO mice showed that GPR40 does not mediate lipotoxicity [29,38]. Thus, it was shown that 3 days of exposure to fatty acids caused a similar inhibition of glucose-induced insulin secretion and reduction of islet insulin content in wild-type and GPR40 KO mice [38].

(iv) In the same study, it was shown that a newly discovered GPR40 agonist restored glucose-induced insulin secretion in β-cells from the neonatal streptozotocin-diabetic rats without any toxic effect [38].

β-Oxidation of Fatty Acids in Mitochondria and Peroxisomes

Fatty acids are an important energy source. They are broken down to acyl-CoA as mainly by β-oxidation at two different subcellular sites; in the mitochondria and in the peroxisomes of higher eukaryotes including all mammals, while in yeast and plants, peroxisomes are the sole site of β-oxidation. The common mechanism comprises four steps: dehydrogenation, hydration, dehydrogenation again and finally thiolytic cleavage for both mitochondrial and peroxisomal β-oxidation. Although similar in mechanism, mitochondrial and peroxisomal β-oxidation differ in substrate specificity and function. In mitochondria, the β-oxidation is tightly coupled to the production of ATP. The resulting NADH and FADH₂ from the β-oxidation, and the following oxidation of acetyl-CoA in the citric acid cycle, are used for ATP production in the respiratory chain. Thus, the complete breakdown of one mol of palmitic acid yields 106 mol ATP [39].

Short- and medium-chain (C4-C8) fatty acids are exclusively β-oxidized in the mitochondria, whereas long-chain (C10-C16) fatty acids are β-oxidized in the mitochondria and the peroxisomes (C14-C16). Very long-chain (C17-C24) fatty acids are handled preferentially by peroxisomes [40]. Other specific substrates for peroxisomal metabolism are, in particular, (i) pristanic acid (2,4,6,10-tetramethylpentadecanoic acid), which is converted by α-oxidation; (ii) di- and trihydroxy-c-6,10-octadecanoic acid; (iii) long-chain dicarboxylic acids, produced by Ω-oxidation of long-chain monocarboxylic acids; (iv) certain polyunsaturated fatty acids, including tetracosaheaxenoic acid (C24:6), which undergoes one cycle of β-oxidation in peroxisomes to produce docosahexaenoic acid (C22:6) and (v) certain prostaglandins and leukotrienes.

Before metabolic degradation in the mitochondria or peroxisomes, transport through the mitochondrial membrane into the organelles is required. In the case of the mitochondria, long-chain fatty acids enter the mitochondrial matrix space via the carnitine cycle [carnitine palmitoyltransferase (CPT)-1/2], whereas short- and medium-chain fatty acids enter directly in their protonated form. Peroxisomal import of fatty acids does not involve the carnitine shuttle; fatty acids are likely transported as acyl-CoA via ABC transporter isoforms (D1–D4) [40]. In the peroxisomal membrane, two specific acyl-CoA synthetases were identified that are responsible for the esterification of fatty acids [41].

In the first reaction of the mitochondrial β-oxidation, acyl-CoA is oxidized by an acyl-CoA dehydrogenase (figure 4) resulting in trans-Δ2-enoyl-CoA. The electrons of this reaction are transferred to FAD and are used in the respiratory chain for ATP production. A group of four acyl-CoA dehydrogenases with different, but overlapping chain length specificities cooperate to facilitate the complete degradation of all fatty acids that can be metabolized by mitochondrial β-oxidation. The chain length specificities of acyl-CoA dehydrogenases provide the basis for metabolic distinction between short chain to medium chain (C₆-C₉), long chain (C₁₀-C₁₆) and very long chain (>C₁₆).

The enzyme enoyl-CoA hydratase adds, in a second reaction, water to trans-Δ2-enoyl-CoA. The resulting product 3-L-hydroxyacyl-CoA is again oxidized through a specific dehydrogenase, which transfers two electrons to NAD⁺. In a final step, the acyl-thioester is hydrolysed and shortens the chain by two carbon atoms. The acyl-CoA can be further degraded by another β-oxidation cycle and the generated acetyl-CoA can be metabolized in the citric acid cycle. The drafted mechanism of the reaction cycle for mitochondrial β-oxidation is in general the same as for peroxisomal β-oxidation, although different isoforms of the enzymes are expressed in the peroxisomes. But there is one fundamental difference in the first step of β-oxidation between mitochondrial and peroxisomal β-oxidation. In the mitochondria, the electrons are transferred to FAD and in the peroxisomes, O₂ is the electron acceptor, which leads to the formation of hydrogen peroxide (H₂O₂). This reaction is catalysed by specific peroxisomal acyl-CoA oxidase (ACOX) isoforms in the rat and in humans. In the rat, extrahepatic peroxisomes contain two ACOXs, including palmitoyl-CoA oxidase (ACOX1) and pristanoyl-CoA oxidase (ACOX3), whereas liver peroxisomes contain an additional cholestanoyl-CoA oxidase (ACOX2). Rat ACOX1 is active with CoA esters of straight-chain mono- and dicarboxylic fatty acids, prostaglandins, very long-chain fatty acids and xenobiotics, whereas rat ACOX3 is active with 2-methyl-branched-chain acyl-CoAs, such as pristanoyl-CoA, but also handles long and very-long straight-chain acyl-CoAs [42]. Interestingly, human peroxisomes contain only two oxidases; the first one is palmitoyl-CoA oxidase, the counterpart of rat ACOX1, with similar substrate spectrum and molecular characteristics [43]. The second human peroxisomal oxidase uses branched-chain fatty acids as substrate, like pristanoyl-CoA and the CoA esters of cholesterol metabolites, as well as straight-chain acyl-CoAs, including the CoA esters of very long-chain fatty acids and dicarboxylic acids [43].

The task of the peroxisomal β-oxidation is a shortening of long-chain fatty acids, which are poor substrates for the mitochondrial β-oxidation. These shortened acyl-CoAs can be released from the peroxisome in a carnitine-dependent manner or as acyl moiety and imported into the mitochondria where they run through β-oxidation for the generation of ATP.
**Oxidative Stress a Cause for Lipotoxicity?**

**Properties of Reactive Species**

According to a rough estimate, 1% of oxygen taken up by mammalian tissues is transformed into reactive oxygen species (ROS). These species can be free radicals, with an unpaired electron, for example $O_2^{•−}$ (superoxide radical) and $OH^•$ (hydroxyl radical), or non-radicals, for example $H_2O_2$. The reactivity of these physiological metabolites is vastly different.

$H_2O_2$ has a relatively long half-life and is therefore the least reactive among these species. It can directly oxidize cysteine groups within proteins, although it is a weak oxidizing agent, which is unable to directly oxidize DNA or lipids [44,45]. The most important intracellular sites of $O_2^{•−}$ (hydroxyl radical), or non-radicals, for example $H_2O_2$. The electron, for example $O_2^{•−}$ (ROS). These species can be free radicals, with an unpaired electron, for example $O_2^{•−}$ (superoxide radical) and $OH^•$ (hydroxyl radical), or non-radicals, for example $H_2O_2$. The reactivity of these physiological metabolites is vastly different.

$O_2$ be induced to produce $O_2^{•−}$ in response $Δp$ is high, therefore forcing back electrons from hydroubiquinone to mitochondrial respiratory chain [48,49]. The mechanism of $O_2^{•−}$ production by isolated complex I is reasonably well understood. Within the complex I, $O_2^{•−}$ can be produced by an electron transfer from the fully reduced FMN (flavine mononucleotide) to $O_2$. The proportion of the FMN that is fully reduced is determined by the NADH:NAD$^{+}$ ratio [48,50]. Inhibition of the respiratory chain by damage, mutation, ischaemia, loss of cytochrome $c$ or by the build-up of NADH because of low ATP demand and subsequent low rate of respiration will increase the NADH:NAD$^{+}$ ratio and lead to a higher rate of $O_2^{•−}$ formation [51–53].

The other mechanism by which complex I produces large amounts of $O_2^{•−}$ is during reverse electron transport (RET) [51,53]. This occurs when the proton motive force ($Δp$) is high, therefore forcing back electrons from hydroubiquinone into complex I. The $O_2^{•−}$ production by RET at complex I was confirmed in isolated brain, heart, muscle and liver mitochondria, under conditions of high $Δp$, with electron supply to the $Q$ pool from succinate, $α$-glycerophosphate or fatty acid oxidation [53–55]. The complex I site, which produces $O_2^{•−}$ during RET, is unclear [50]. The most simple possibility is that RET forces electrons right back through complex I to the FMN, and that the site of $O_2^{•−}$ production is the same during RET as it is for $O_2^{•−}$ production from the reduced FMN in response to an elevated NADH:NAD$^{+}$ ratio [50,53]. A second site of $O_2^{•−}$ formation could be the ubiquinone (Q)-binding site in complex I [56].

Complex III, which funnels electrons from the $Q$ pool to cytochrome $c$, has for a long time been regarded as a source of $O_2^{•−}$ within mitochondria [48,57]. Although complex III can be induced to produce $O_2^{•−}$ from ubisemiquinone in a reaction with $O_2$ bound to the $Q_0$ site, its production in mitochondria is far lower and negligible compared with the maximal rates of $O_2^{•−}$ production from complex I. Whereas $O_2^{•−}$ generated at complex I is released to the matrix site, it can be released from complex III to both sides of the inner membrane [51,54].

Apart from the induction of electron flux in the electron transport chain by fatty acid metabolism, there are also direct interactions possible between NEFAs and the respiratory chain proteins resulting in an increased $O_2^{•−}$ production [45]. Especially polyunsaturated NEFAs partially inhibit electron transport within complexes I and III, thereby facilitating the ‘electron leak’ and enabling one-electron reduction of $O_2$ to $O_2^{•−}$. According to a current view, respiratory chain complexes interact with each other, thereby forming a supercomplex known as respirasome [58]. An advantage of respirasome formation can be electron channelling to avoid ROS formation [58]. Keeping this in mind, it might be that NEFAs destabilize the respirasome, thereby facilitating ROS production. It has also been shown that NEFAs can cause detachment of cardiolipin-bound cytochrome $c$ from the outer side of the inner membrane [45,59], thus further impeding electron transport along the respiratory chain and increasing the reduction state of upstream electron carriers, which has been shown to dramatically increase ROS production in rat heart and brain [52].

It has been reported that incorporation of fatty acids into phospholipid model membranes or mitochondrial membranes can alter membrane fluidity [60,61]. Erucic acid, for example, increases membrane fluidity, which is accompanied by an increase in $O_2^{•−}$ production [60]. However, the mechanism by which increased fluidity of the inner membrane lipid core may facilitate the ‘electron leak’ and one-electron reduction of $O_2$ remains to be elucidated.

In contrast to the NEFA-mediated enhancement of ROS production, the protonophoric activity of NEFAs can abolish RET-dependent $O_2^{•−}$ generation, which has been shown with mitochondria from rat heart and brain [62,63]. This is based on the protonophoric activity (‘mild uncoupling’) of NEFAs caused by a cyclic movement of protonated and deprotonated forms of fatty acids across the inner mitochondrial membrane.

**Fatty Acids as Activators of Plasma Membrane NADPH Oxidase**

Phagocytic cells of the immune system, such as macrophages, contain a membrane-associated enzyme complex, termed NADPH oxidase. This is another source of $O_2^{•−}$ generation. These cells produce $O_2^{•−}$ in order to damage and kill pathogenic organisms. Besides the immune system, NADPH oxidase (isoforms 1–3) is also expressed in various other tissues including pancreatic islets [64]. This enzyme consists of six hetero subunits, which associate in a stimulus-dependent manner to catalyse the one-electron reduction of $O_2$ to generate $O_2^{•−}$ utilizing NADPH as electron donor. As $β$-cells have a relatively low pentose phosphate pathway activity, NADPH could be generated by malic enzyme catalysed conversion of malate to pyruvate [65]. The two subunits gp91$^{phox}$ and p22$^{phox}$ of NADPH oxidase are integral membrane proteins, which form together with the flavocytochrome $b_558$ the catalytic core. The additional subunits, namely p67$^{phox}$, p47$^{phox}$, p40$^{phox}$...
and the small GTPase Rac, are required for the regulation and are located in the cytosol during the resting state [66]. Enzyme activation is initiated by p47phox phosphorylation through various protein kinases, for example protein kinase C (PKC), promoting the subsequent translocation of the cytosolic subunits to the membrane [66,67]. It has been reported that nutrients, such as high levels of glucose and palmitic acid, stimulated aortic smooth and endothelial cell phagocyte-like NADPH oxidase via PKC-dependent activation [67]. Recently, an increased production of the NADPH oxidase components gp91phox and p22phox was shown in β-cells from animal models of type 2 diabetes [68]. p47phox expression was also found in a clonal rat pancreatic β-cell line, BRIN BD11 [65]. In this cell line, the protein production of p47phox was elevated after 24 h treatment with palmitic acid, whereas the polyunsaturated NEFA arachidonic acid did not increase p47phox protein production [65]. The increased production and activity of NADPH oxidase by palmitic acid may result in excessive O2•− production [66]. The precise mechanisms for participation of PKC in the activation of NADPH oxidase and the physiological role of this enzyme in pancreatic β-cells still remain to be fully elucidated.

**Peroxisomally Generated Hydrogen Peroxide as a Mediator of Lipotoxicity**

NEFA toxicity is strongly structure dependent, thus only long-chain and very long-chain (>13) saturated NEFAs exhibit a strong cytotoxic effect upon insulin-producing cells. Experimental evidence showed that elevated production of ROS could mediate NEFA-induced toxicity. When NEFAs enter the cell, they can be metabolized by β-oxidation in two different subcellular compartments as described earlier. In the mitochondria, the breakdown of NEFAs is paralleled by an elevated O2•− formation as a by-product in the respiratory chain. For mitochondrial β-oxidation, short-chain NEFAs as well as medium- to long-chain NEFAs serve as substrates. This pathway, therefore, cannot explain the strong cytotoxicity especially of long-chain NEFAs. By contrast, the peroxisomal β-oxidation prefers long-chain and very long-chain NEFAs as substrates, which is in accordance with their toxicity profile. Additional evidence for the involvement of peroxisomes arises from a viability analysis of insulin-producing RINm5F cells in the presence of pristanic acid, which is exclusively metabolized as mediators of lipotoxicity. In the first step of peroxisomal β-oxidation, FAD-containing ACOX 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 H2O2 [69], as described earlier. Remarkably, in rat liver, for example, around 20% of total oxygen consumption is accounted for peroxisomal oxidase activity [70], producing 35% of total cellular H2O2 [71]. This organelle is therefore one of the main sites in the cell, where ROSs are both generated and scavenged. Peroxisomes are single membrane-bound, highly dynamic organelles present in virtually every eukaryotic cell. Immunofluorescence analyses of hepatoma cells (HepG2) and rat β-cells showed a comparable abundance of about 130 peroxisomes per cell (Elsner et al., in prep.). The biogenesis of peroxisomes involves either fission of pre-existing organelles or budding from the ER [69]. The peroxisomes contain the enzymes for fatty acid α-oxidation, β-oxidation of long-chain NEFAs, purine catabolism and biosynthesis of glycerolipids and bile acids [40]. The oxidation of these various substrates is performed by O2- consuming oxidases that produce H2O2 as a by-product. For the detoxification of H2O2, the oxidoreductase catalase, which has a high turnover rate, is expressed in the peroxisomes of most tissues but not in those of pancreatic β-cells (figure 5) [72,73].

To elucidate the role of different ROS and their intracellular site of formation, we used RINm5F insulin-producing cells overexpressing various antioxidative enzymes. The H2O2-detoxifying enzyme catalase was either overexpressed in the peroxisomes/cytosol or by fusing to the mitochondrial-targeting sequence in the mitochondria. The formation of superoxide radicals was analysed by overexpressing the cytosolic copper zinc superoxide dismutase (CuZnSOD) or the mitochondrial isofrom manganese superoxide dismutase (MnSOD). These enzymes catalyse the conversion of O2•− into O2 and H2O2. The latter is degraded by catalase. Experiments with those cells showed that only peroxisomal/cytosolic catalase provided protection against palmitic acid-induced toxicity; mitochondrial catalase was not protective in the MTT viability assay. The superoxide dismutase (SOD) isoenzymes MnSOD and CuZnSOD mediated no protection against palmitic acid-induced toxicity, indicating that the formation of superoxide...
radicals does not play such a crucial role in lipotoxicity as does the formation of H₂O₂ (Elsner et al., in prep.). This is supported also by the observation that a combined overexpression of SOD and catalase provided no further protection (unpublished observation). Using the H₂O₂-sensitive fluorescence protein HyPer as a novel, specific method for detecting H₂O₂, we clearly identified H₂O₂ as the main ROS formed during palmitic acid treatment. To determine the subcellular site of H₂O₂ formation, the HyPer protein was fused to a peroxisome- or a mitochondrion-targeting sequence to allow organelle-specific expression. These experiments showed that peroxisomes were indeed the major site of H₂O₂ formation in insulin-producing cells, whereas the mitochondria were a site of minor contribution (Elsner et al., in prep.). The detected H₂O₂ formation due to palmitic acid exposure provides experimental evidence that H₂O₂ generated in the first step of peroxisomal β-oxidation is of particular importance in long-chain NEFA-mediated toxicity (figure 3).

We thus suggest that mitochondrial β-oxidation may not be able to cope with the elevated levels of long-chain NEFAs, such as palmitic acid, that are associated with obesity and type 2 diabetes. This overload results in a larger proportion of fatty acids being metabolized through peroxisomal β-oxidation, thereby leading to an increased formation of toxic H₂O₂.

**Conclusion**

While GPR40 plays a decisive role in the potentiation of glucose-induced insulin secretion by saturated and unsaturated long-chain NEFAs, GPRs do not mediate lipotoxicity to insulin-secreting cells. Lipotoxicity is ROS mediated. The responsible reactive species is not the O₂•− radical but rather H₂O₂. Subcellular compartmentation of ROS generation is of crucial importance for lipotoxicity to pancreatic β-cells in diabetes. Although the ultimate reactive species responsible for β-cell death is likely to be always the highly toxic OH• radical, the initial reactive species responsible for lipotoxicity in type 2 diabetes is H₂O₂ generated in peroxisomal β-oxidation. This is at variance from cytokine-mediated β-cell death in type 1 diabetes where H₂O₂ is formed from the O₂•− radical generated in mitochondrial metabolism that, in conjunction with an interaction with NO•, is responsible for the cytotoxicity [44]. O₂•− radicals generated in mitochondrial metabolism or by NADPH oxidase at the plasma membrane are unlikely to be involved in lipotoxicity. Thus, while peroxisomal stress prevails in lipotoxicity, it is mitochondrial stress in cytokine-mediated toxicity. Both forms of oxidative stress go along with an increased pressure upon protein synthesis and folding, causing an unfolded protein response (UPR) in the pancreatic β-cells [74], which explains ER stress both in lipotoxicity and cytokine-mediated toxicity [75,76].

Mitochondrial and peroxisomal stress as the underlying causes, both characterized by an increased generation of toxic ROS, are responsible in type 1 and type 2 diabetes for β-cell dysfunction and ultimately death of the pancreatic β-cell, which is particularly vulnerable [44] because of its low antioxidative defense status [72,73].

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**Conflict of Interests**

The authors do not declare any conflict of interest relevant to this manuscript.

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