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REVIEW

Oxidative stress and calcium dysregulation by palmitate in type 2 diabetes

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Free fatty acids (FFAs) are important substrates for mitochondrial oxidative metabolism and ATP synthesis but also cause serious stress to various tissues, contributing to the development of metabolic diseases. CD36 is a major mediator of cellular FFA uptake. Inside the cell, saturated FFAs are able to induce the production of cytosolic and mitochondrial reactive oxygen species (ROS), which can be prevented by co-exposure to unsaturated FFAs. There are close connections between oxidative stress and organellar Ca²⁺ homeostasis. Highly oxidative conditions induced by palmitate trigger aberrant endoplasmic reticulum (ER) Ca²⁺ release and thereby deplete ER Ca²⁺ stores. The resulting ER Ca²⁺ deficiency impairs chaperones of the protein folding machinery, leading to the accumulation of misfolded proteins. This ER stress may further aggravate oxidative stress by augmenting ER ROS production. Secondary to ER Ca²⁺ release, cytosolic and mitochondrial matrix Ca²⁺ concentrations can also be altered. In addition, plasmalemmal ion channels operated by ER Ca²⁺ depletion mediate persistent Ca²⁺ influx, further impairing cytosolic and mitochondrial Ca²⁺ homeostasis. Mitochondrial Ca²⁺ overload causes superoxide production and functional impairment, culminating in apoptosis. This vicious cycle of lipotoxicity occurs in multiple tissues, resulting in β -cell failure and insulin resistance in target tissues, and further aggravates diabetic complications.

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INTRODUCTION

Free fatty acids (FFAs) are important sources of fuel required for efficient cellular energy production. FFAs enter mitochondria via carnitine palmitoyltransferase 1 (CPT1) and undergo β -oxidation to generate acetyl-CoA, which serves as a substrate for the Krebs cycle. Fatty acid metabolism generates reducing equivalents used by the electron transport chain (ETC) for ATP synthesis.¹ Increased β -oxidation attenuates further mitochondrial FFA uptake through the formation of malonyl CoA, an inhibitor of CPT1. Excess FFA critically induces reactive oxygen species (ROS) generation, resulting in lipotoxicity associated with ER stress, calcium dysregulation, mitochondrial dysfunction and cell death.

Palmitate, stearate and oleate are the most abundant FFAs, accounting for 70–80% of total plasma FFAs.² FFA concentrations in patients with type 2 diabetes are significantly higher than in healthy subjects.^{3,4} Compared with normal subjects, rates of palmitate appearance in plasma are 1.5- and 3-fold

higher in type 2 diabetic individuals during nocturnal and postprandial states, respectively.⁴ In the Paris Prospective Study, increased plasma FFA concentration and decreased 2-h plasma insulin levels are considered to be independent predictors of type 2 diabetes in subjects with a history of impaired glucose tolerance. Among impaired glucose tolerance subjects who develop type 2 diabetes, 78% are in the highest tertile of fasting FFA concentrations. It has been suggested that lipotoxicity is associated with uncompensated insulin secretion in patients with insulin resistance, leading to overt type 2 diabetes.⁵

In this review, we summarize the molecular mechanisms leading to palmitate-induced toxicity in type 2 diabetes, including sources of ROS generation and Ca²⁺-mediated pathogenic changes. These mechanisms show harmful cross-interactions. Endoplasmic reticulum (ER) Ca²⁺ release due to palmitate-induced oxidative stress results in cytosolic and mitochondrial Ca²⁺ overload, which may further accelerate

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ROS generation from mitochondria and facilitate permeability transition (PT) pore opening. The activation of store-operated Ca²⁺ (SOC) entry triggered by ER Ca²⁺ depletion augments the persistent Ca²⁺ load. The interruption of such vicious cycles of ROS formation and Ca²⁺ dysregulation may be a good therapeutic target for the prevention and treatment of metabolic diseases related to lipotoxicity.

CD36: FATTY ACID TRANSPORTER OR RECEPTOR?

CD36 is an 88-kDa, ditopic, heavily N-linked glycosylated transmembrane protein that is also known as fatty acid translocase (FAT).⁶ CD36 is abundantly expressed in tissues with a high capacity for fatty acid metabolism (for example, adipose tissue, cardiac and skeletal muscles).^{6–8} Other cells and tissues including liver,⁹ endothelial cells,¹⁰ monocytes, macrophages,^{11,12} pancreatic β -cells¹³ and podocytes¹⁴ also express CD36.

Muscle-specific over-expression of CD36 enhances FFA uptake and thus decreases plasma triglyceride and fatty acids levels.¹⁵ Conversely, FFA uptake is impaired in CD36 null mice with high plasma concentrations of cholesterol and triglyceride.¹⁶ CD36 expression is low in normal hepatocytes and does not have a significant role in FFA uptake.^{8,9,17–19} The Pro90Ser CD36 mutation in humans perturbs the FFA uptake of muscle and adipose tissue, but hepatic uptake is not affected under suppressed or slightly increased concentrations of palmitate.¹⁸ Consistently, hepatic FFA uptake is not disturbed in CD36 knockout mice.⁸ Under a high-fat diet or in hepatic steatosis, CD36 is highly inducible by activation of nuclear receptors, including liver X receptor, pregnane X receptor, peroxisome proliferator-activated receptor γ and the aryl hydrocarbon receptor.^{9,17,19} However, controversies arise concerning the impact of CD36 on fatty liver disease. Hepatocyte-specific CD36 disruption significantly reduces hepatic triacylglycerol, diacylglycerol (DAG) and cholesterol ester content and improves insulin sensitivity when a high-fat diet is consumed.¹⁹ However, liver-specific CD36 overexpression attenuated hepatic steatosis and insulin resistance in another study with transgenic mice.^{17,19}

In addition to its role in FFA transport, CD36 has an important role in signal transduction through the activation of non-receptor tyrosine kinases of the Src family, including Fyn and Lyn.^{20,21} The binding of long chain (LC)-FFAs to CD36 stimulates the tyrosine phosphorylation of downstream proteins, inducing pro-inflammatory and atherogenic responses associated with diabetes, atherosclerosis, thrombosis, and Alzheimer disease.²⁰ Ligand binding to CD36 also stimulates phospholipase C (PLC) and, as a consequence, IP₃-mediated ER Ca²⁺ release. This signaling pathway contributes, for example, to the sensing of LC-FFA in taste buds.²² In addition, CD36 stimulates SOC influx. The associated increase in Ca²⁺ activates Ca²⁺-dependent phospholipase A2 and prostaglandin synthesis involved in inflammatory responses.²¹

Interestingly, CD36 is upregulated in response to high glucose in insulin-secreting cells and in patients with diabetic nephropathy. Such regulation of CD36 expression may lead to

the exacerbation of glucolipotoxicity via increased FFA uptake.^{23,24} In insulinoma cells, CD36 induction increases the uptake of FFA, leading to the blunting of the functional interplay between glucose and lipids in insulin secretion as a consequence of impaired oxidative metabolism.²⁵ The disruption of the *CD36* gene, however, protects from obesity-associated steatosis and insulin resistance.²⁶ In diabetic animals, a lack of CD36 attenuates NADPH oxidase (NOX)-dependent ROS generation. Moreover, the targeted disruption of CD36 in macrophages shows protective action against atherosclerosis.²⁷ Therefore, CD36 could be a therapeutic target for the treatment of metabolic dysfunction worsened by dyslipidemia.

Sulfo-N-succinimidyl derivatives have been developed as selective inhibitors for CD36.^{28,29} Preincubation with a CD36 inhibitor prevents saturated FFA-induced ROS production and cytotoxicity.^{24,30} Sulfo-N-succinimidyl derivatives also inhibit oxidized low-density lipoprotein (oxLDL) uptake in macrophages.²¹ Recently, Souza *et al.*³¹ demonstrated that the 5A peptide antagonizes oxLDL binding to CD36, inhibiting inflammation and oxidative stress in vascular tissues. The 5A peptide, through its inhibition of CD36, also reduces glomerular injury and tubule-interstitial fibrosis in animal models of chronic kidney disease.³¹

OXIDATIVE STRESS INDUCED BY FATTY ACIDS

Reactive oxygen species are essential signaling molecules that regulate physiological cell functions.³² However, the overproduction of ROS in pathologic conditions has detrimental consequences, causing organellar stress, injury and cell death.^{33,34} Palmitate is a potent inducer of ROS in a number of cell types, including pancreatic β cells,^{35–37} cardiomyocytes,^{34,38} vascular smooth muscle cells,³⁹ endothelial cells,⁴⁰ skeletal muscle cells,⁴¹ glomerular podocytes,³⁰ hepatocytes⁴² and adipocytes.⁴³ CD36 appears to be required for fatty acid-induced ROS production due to the fact that the knockdown of CD36 prevents palmitate-dependent oxidative stress.²³

Increased mitochondrial fatty acid oxidation has been proposed as the main process leading to ROS generation in lipotoxicity (Figure 1). The oxidation of palmitate delivers excess electrons to the ETC, which thus causes superoxide overproduction.^{44–46} There are, however, conflicting data in the literature showing that the acceleration of β -oxidation actually relieves oxidative stress, and the inhibition of mitochondrial fatty acid uptake aggravates ROS production.^{47,48} The molecular mechanisms for cellular ROS generation by palmitate, therefore, remain to be fully elucidated.

Palmitate-induced superoxide cannot be fully eliminated by the addition of the complex III inhibitor antimycin A, revealing that ROS are also generated through sources other than the ETC.⁴⁴ In chondrocytes, a mixture of oleate and palmitate enhances ROS production and induces cell apoptosis, mainly by upregulating the protein levels of NOX4.⁴⁹ Notably, NOX4 is expressed in mitochondria and contributes to mitochondrial ROS production.^{50,51} A recent study suggests that the activation of protein kinase C α (PKC α) by palmitate increases ROS production through NOX2 upregulation in cardiomyocytes.³⁸

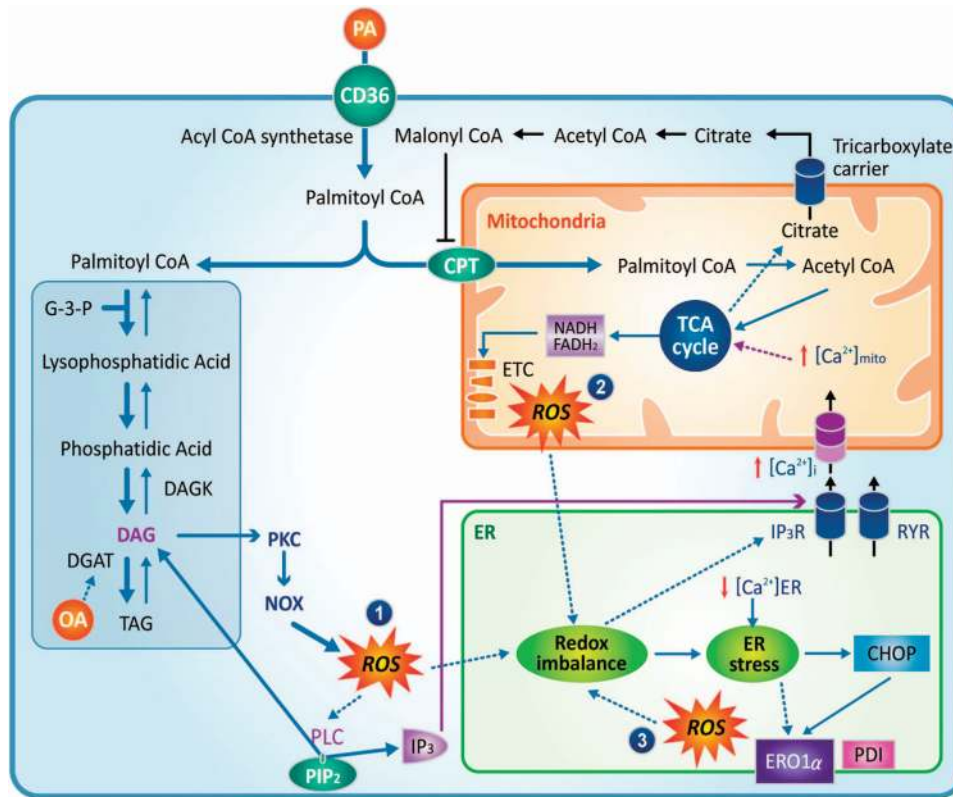


Figure 1 Palmitate induces ROS overproduction. (1) Increased β -oxidation, (2) DAG-PKC-NOX, (3) CHOP-ERO1 α and PDI under ER stress. ROS produced by palmitate triggers PLC activation, ER Ca²⁺ release, ER stress and mitochondrial dysfunction, which, in turn, aggravate ROS generation. CHOP, CCAAT-enhancer-binding protein homologous protein; DAG, diacylglycerol; ERO1 α , ER oxidoreductin 1 alpha; NOX, NADPH oxidase; PDI, protein disulfide isomerase; PKC, protein kinase C; PLC, phospholipase C; ROS, reactive oxygen species.

An upstream stimulus of PKC is DAG, which is produced either in a membrane-delimited manner with G_{q/11}-coupled PLC or by enzymatic synthesis from phosphatidic acid. Palmitate increases DAG in a number of cell types.^{52–54} The formation of this signaling molecule may be responsible for palmitate-induced PKC activation and ROS generation (Figure 1). Moreover, there is crosstalk between mitochondria and the NADPH oxidase system via feed-forward amplification of ROS production.⁵⁵ The involvement of ER oxidoreductin 1 alpha (ERO1 α) and disulfide isomerase during ER stress, as well as ER-mitochondrial Ca²⁺ dysregulation in ROS overproduction, will be discussed later in this review.

Unlike palmitate, oleate is an unsaturated fatty acid with a *cis* double bond at position 9. Oleate may stimulate ROS generation but may also protect from oxidative stress. Oleate has been reported to increase intracellular H₂O₂ production in rat smooth muscle cells,⁵⁶ pancreatic β -cells,⁵⁷ and human hepatoma HepG2 cells.⁵⁸ Other studies, however, reported no effect of oleate on ROS generation in smooth muscle cells from the human coronary artery⁵⁹ or Chang liver cells.⁶⁰ Oleate is even able to attenuate or abolish palmitate-induced ROS synthesis when the two fatty acids are used in combination.^{30,61} Despite apparently conflicting data, there is some convincing evidence that oleate does not increase mitochondrial ROS level when employing a technique specifically detecting mitochondrial

ROS.^{38,61} Reduced ROS generation in the presence of oleate is correlated with a protective effect of unsaturated FFAs on ER stress and cytotoxicity.

PALMITATE INDUCES ER STRESS

Approximately one-third of all newly synthesized proteins are imported into the ER.⁶² Proteins trafficking through the ER undergo post-translational processing modifications, including glycosylation and chaperone-assisted protein folding. The oxidative folding process, especially the generation of disulfide bonds, generates a large amount of ROS.⁶³ Therefore, redox homeostasis is vital to maintain ER folding capacity. Palmitate-induced ROS formation impairs ER redox status and leads to the accumulation of misfolded or unfolded proteins.^{64,65} The associated excess workload beyond the protein folding capacity of the ER activates the unfolded protein response in an attempt to reestablish normal ER function.⁶⁶ Unfolded protein response-dependent signaling is initiated by three ER transmembrane proteins: inositol-requiring protein 1 α (IRE1 α), protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6). These stress sensors normally bind to a luminal ER chaperone called the binding immunoglobulin protein (BiP or GRP78). BiP has a high affinity for unfolded proteins. As unfolded proteins accumulate in the ER lumen, BiPs detach from the stress sensors activating downstream signaling, leading to three main outcomes:

(1) the overall attenuation of translation, with the simultaneous (2) promotion of the translation of ER chaperones and (3) the restoration of the ER-associated degradation (ERAD) system.^{67,68} If the stress is too severe to be resolved by the unfolded protein response, the cell triggers a death program to be eliminated.

The condensation of palmitoyl-CoA, the activated form of palmitate and serine, is the first step in the biosynthesis of ceramide, which is catalyzed by serine palmitoyltransferase. Ceramide activates protein phosphatase 2A and PKC, both of which can inhibit Akt activation, leading to insulin resistance in skeletal muscle and adipose tissue.^{69,70} This pathogenic process activates pro-apoptotic signaling and cytochrome *c* release from mitochondrial inter-membrane space.⁷¹ Ceramide also inhibits mitochondrial beta-oxidation, which aggravates palmitate-induced lipotoxicity.⁷² Intriguingly, ceramide induces the loss of the ER calcium pool and ER stress. The inhibition of the sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA) by ceramide has been suggested as the main mechanism of ER calcium depletion.⁷³

Unlike palmitate, oleate does not cause a significant ER stress response.^{30,38,61} Moreover, oleate prevents palmitate-induced ER stress, c-Jun N-terminal kinase (JNK) activation and cell death,^{74,75} all of which are consistent with reduced ROS

generation. A key difference between the two fatty acids is that oleate, but not palmitate, activates diacylglycerol acyl transferase (DGAT). The stimulation of DGAT lessens DAG accumulation by converting it to triacylglycerol.^{76–78} Using ³H-labeled palmitate, it was shown that oleate attenuates palmitate-induced DAG formation and instead leads to the preferential accumulation of triacylglycerol.⁷⁹ Oleate promotes the mitochondrial oxidation of palmitate by increasing CPT1 expression. This mechanism contributes to diminished total palmitate and palmitate-derived toxic metabolites.⁷⁸

The ER stress response could be a therapeutic target to prevent palmitate-induced lipotoxicity. There have been attempts to tackle diseases of protein misfolding, such as cystic fibrosis, α 1 antitrypsin deficiency, Alzheimer disease and type 2 diabetes, using the chemical chaperone 4-phenylbutyric acid.^{80–83} Taurine-conjugated ursodeoxycholic acid (TUDCA) has also been tested as a chaperone to protect hepatocytes from palmitate-induced ER stress and apoptosis.⁸⁴ Salubrinal, a selective chemical inhibitor of eIF2 α phosphatase, was introduced to prevent ER stress.⁸⁵ Further studies revealed, however, that salubrinal treatment shows deleterious effects in pancreatic β -cells and other cell types.^{86,87}

Several studies have demonstrated that knockdown of ER stress proteins (for example, CCAAT-enhancer-binding protein

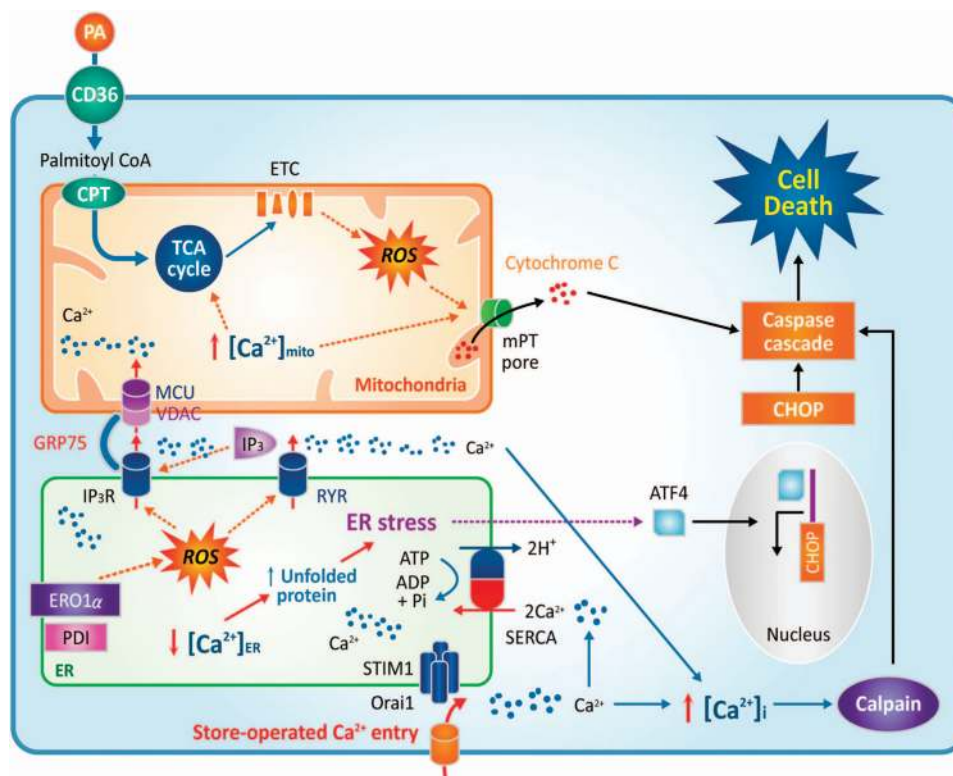


Figure 2 Palmitate disturbs intracellular Ca²⁺ homeostasis. ROS activate IP₃R and RYR, which release Ca²⁺ from the ER. The deprivation of ER Ca²⁺ leads to ER stress and CHOP upregulation. Ca²⁺ is transported into mitochondria through a specialized structure composed of IP₃R, VDAC, MCU and GRP75. Excessive Ca²⁺ in mitochondria leads to cytochrome *c* release. SOC entry triggered by ER Ca²⁺ depletion elicits the persistent influx of Ca²⁺ into cytosol and mitochondria. High intracellular calcium activates calpain signaling. Cytochrome *c*, CHOP and calpain all provoke caspase activation and cell death. CHOP, CCAAT-enhancer-binding protein homologous protein; GRP75, 75 kDa glucose-regulated protein; MCU, mitochondrial Ca²⁺ uniporter; ROS, reactive oxygen species; SOC, store-operated Ca²⁺; VDAC, voltage-dependent anion channel.

homologous protein (CHOP)) has protective effects on palmitate-induced apoptosis in insulin-secreting cells,^{68,88} podocytes⁷⁵ and other cell types.^{84,89} However, CHOP knockout mice suffer from steatohepatitis and fibrosis due to the pro-inflammatory actions of CHOP-deleted macrophages in the liver. Therefore, more research is required to find better interventions to prevent palmitate-induced ER stress without serious adverse events.

ER CALCIUM DEPLETION BY PALMITATE

Luminal ER Ca²⁺ concentration is particularly important for protein folding. High levels of Ca²⁺ in the ER lumen (>400 μM) are required for interactions among ER chaperones and between chaperones and unfolded proteins.⁶⁶ SERCA maintains high ER Ca²⁺ concentrations. A chronic reduction of ER Ca²⁺ stores elicits the accumulation of unfolded or misfolded proteins and initiates an ER stress response (Figure 2).^{66,90} Exposure to thapsigargin, an inhibitor of SERCA, is applied to induce ER stress experimentally by depleting the ER Ca²⁺ stores. Palmitate-induced ER stress is also associated with a sustained reduction of the ER Ca²⁺ pool, which has been demonstrated directly through cytosolic and ER Ca²⁺ measurements.^{30,68,88,91} ER Ca²⁺ loss caused by FFA triggers the unfolded protein response to rescue cells from misfolded protein overload or programmed cell death.⁹²

ER proteome analysis in the liver of ob/ob mice shows a fundamental shift in ER function in obesity from protein synthesis to lipid synthesis and metabolism.⁹³ One important factor inducing ER calcium depletion in obesity is the increased phosphatidylcholine/phosphatidylethanolamine ratio, which disrupts ER calcium refilling capacity by inhibiting SERCA activity. This regulation did not occur at the level of expression, as the SERCA protein was slightly more abundant in ob/ob mice compared to lean mice. The suppression of phosphatidylcholine synthesis from phosphatidylethanolamine normalized the phosphatidylcholine / phosphatidylethanolamine ratio, protected against ER stress and improved systemic glucose homeostasis.

The accumulation of misfolded proteins causes ROS generation from the oxidative folding machineries in the ER and mitochondria.⁹⁴ Defective disulfide bond formation depletes glutathione in the ER and produces oxygen radicals via ERO1α and protein disulfide isomerase.⁶³ Intriguingly, ROS produced by ERO1α activates type 1 IP₃ receptors (IP₃R) and stimulates ER Ca²⁺ release.⁹⁵ Consequent ER Ca²⁺ loss further deteriorates the protein-folding process and augments ROS generation. Furthermore, prolonged ER stress increases CHOP expression, which upregulates ERO1α, causing additional oxidative stress. This positive feedback mechanism amplifies oxidation-triggered IP₃R activation and ER Ca²⁺ release (Figure 1). Blocking this vicious cycle between ER ROS formation and ER Ca²⁺ release could be a pertinent therapeutic strategy. In support of this approach, we observed that palmitate-induced ER Ca²⁺ loss was prevented by both ROS scavengers or the inhibition of IP₃ generation.³⁰

It is noteworthy that H₂O₂-mediated oxidative stress can activate PLCγ and generate IP₃ and DAG in astrocytes and lung endothelial cells.^{96,97} Consistent with these findings, PLC activation was observed in podocytes treated with either palmitate or H₂O₂. Pretreatment with a PLC inhibitor attenuated palmitate-induced ER Ca²⁺ loss, suggesting that IP₃ generation from phosphatidylinositol 4,5-bisphosphate (PIP₂) contributes to ER Ca²⁺ release via the IP₃R.³⁰ In addition, DAG, the other signaling molecule produced by PLC activity, may also participate in palmitate-dependent ER Ca²⁺ loss. This hypothesis was supported by experimental evidence showing that palmitate-induced ER Ca²⁺ depletion and ER stress were surprisingly augmented by treatment with a DAG kinase blocker, leading to DAG accumulation.³⁰ DAG accumulation activates PKCδ.⁷⁴ PKC activity upregulates NOX (Figure 1), and more ROS are thus generated, as discussed earlier, leading to further ER Ca²⁺ loss. The inhibition of PKC blunted the effect of palmitate on ER Ca²⁺, suggesting a critical pathogenic role for DAG-mediated PKC activation.³⁰ We propose a synergistic stimulation of Ca²⁺ release from the ER by IP₃ and DAG, although further detailed studies are required to substantiate our working model.

PALMITATE DISTURBS INTRACELLULAR CALCIUM HOMEOSTASIS

Plasma membrane Ca²⁺ ATPase (PMCA) and SERCA establish 1000- to 10 000-fold change gradients of Ca²⁺ concentrations across the plasma membrane and the ER membrane.⁹⁰ Therefore, inappropriate and uncontrolled cytosolic Ca²⁺ increases that result from Ca²⁺ influx from the extracellular space or release from the ER are a burden for the cell as it tries to maintain intracellular Ca²⁺ homeostasis. Ca²⁺ stress may initiate pathogenic processes such as calpain-mediated cell death. In β-cells, for instance, it was observed that palmitate-induced ER Ca²⁺ release activates the calcium-dependent pro-apoptotic protease calpain-2.⁹²

Ca²⁺ release from the ER participates in cell death mechanisms. The luminal ER Ca²⁺ level is an important factor determining susceptibility to apoptosis triggered by different kinds of proapoptotic stimuli, including ceramides and arachidonic acid.^{98,99} Recent discoveries support these observations by revealing a role for ER-mitochondrial contacts, known as the mitochondria-associated ER membrane (MAM) in apoptosis (Figure 2). MAMs are specialized sub-compartments of the ER where the distance between the ER and the mitochondrial membranes is <25 nm.¹⁰⁰ MAMs have been reported as either larger or tighter in diabetic mice on a high-fat diet.^{101,102} The physical contact points between the ER and mitochondria are enriched for specific membrane proteins such as the IP₃ receptor, the voltage-dependent anion channel and the mitochondrial Ca²⁺ uniporter (MCU). Additional adaptor proteins, including GRP75, are also required to establish high capacity Ca²⁺ transfer from the ER to the mitochondria.¹⁰³ The increased density of MAMs in the cells of animals fed a high-fat diet may aggravate ER Ca²⁺ depletion

and mitochondrial Ca²⁺ overload, although this hypothesis requires further experimentation.¹⁰⁴

The activation of Ca²⁺ signals via PLC-mediated IP₃ generation depletes ER Ca²⁺ and may thus have a negative impact on ER function and cell survival.⁹⁰ To prevent this pathogenic consequence, there is an innate response to refill the ER Ca²⁺ reservoir. In the ER membrane, the stromal interaction molecule (STIM), a transmembrane protein with luminal EF hands, senses ER Ca²⁺ levels. A decrease in ER Ca²⁺ leads to STIM translocation to the plasma membrane-ER junctions. In these sub-plasma membrane areas, STIM proteins oligomerize to form clusters to recruit Orai1, a plasmalemmal Ca²⁺ channel. Orai1 mediates SOC entry until ER Ca²⁺ stores are refilled, at which point STIM oligomers again become dispersed. Notably, palmitate-treated cells maintain STIM1 oligomerization, signifying that ER Ca²⁺ release and depletion of stores persist.³⁰ Upon extracellular Ca²⁺ addition, palmitate-treated cells show strong and sustained increases in cytosolic Ca²⁺, whereas there is a negligible influence on Ca²⁺ influx in control cells (Figure 2).³⁰ This evidence suggests that ER Ca²⁺ depletion by palmitate induces sustained SOC entry, which may raise cytosolic and mitochondrial Ca²⁺ to an intolerable level. Low extracellular Ca²⁺ conditions protect against palmitate-induced cytotoxicity, suggesting that SOC contributes to the harmful effects of palmitate.¹⁰⁵ It should be noted that Ca²⁺ influx via SOC entry is essential for physiological process such as immune cell activation. Moreover, there have been no reports of a truly selective SOC inhibitor until now. Nevertheless, we suggest that the prevention of sustained SOC activation could be a candidate for therapeutic targets to prevent lipotoxicity.

MITOCHONDRIAL DYSFUNCTION BY PALMITATE

Mitochondria have an essential role in energy metabolism, biosynthetic processes, Ca²⁺ homeostasis and the integration of apoptotic signals.^{106,107} Ca²⁺ in the mitochondrial matrix and extramitochondrial locations modulates mitochondrial functions, including intermediary metabolism and ATP synthesis. Mitochondrial Ca²⁺ activates pyruvate dehydrogenase, Krebs cycle activity, mitochondrial transporters, and ATP synthase.^{108–110} MCU is the main molecule responsible for mitochondrial Ca²⁺ uptake and the activation of mitochondrial metabolism.^{111,112} Unexpectedly, no obvious phenotype was initially observed in mice lacking MCU.¹¹³ In animals with a cardiac muscle-specific deletion, however, MCU deficiency induces defects in acute metabolic stimulation and protects against ischemia-reperfusion injury.¹¹⁴ Local increases in cytosolic Ca²⁺ arrest the movement of mitochondria, allowing the organelle to efficiently take up and sequester Ca²⁺ into its matrix to stimulate mitochondrial energetics.¹¹⁵ Excess mitochondrial Ca²⁺ uptake, in contrast, induces mitochondrial permeability transition pore opening, followed by cytochrome *c* release and apoptotic cell death (Figure 2).¹¹⁶

Accumulating evidence suggests that human subjects with obesity or insulin resistance exhibit reduced oxygen consumption rates, decreased expression of mitochondrial proteins, and

impaired ATP synthesis.^{117,118} Mitochondrial dysfunction decreases β -oxidation and may elevate plasma FFA concentration, thereby aggravating lipotoxicity. The supplementation of tricarboxylic acid cycle substrates to facilitate mitochondrial FFA metabolism rescues lipotoxicity in insulin-secreting cells.^{47,119} However, excessive FFA in mitochondria stimulates superoxide generation from the ETC, leading to cytotoxicity.⁴⁴

Mitochondria are dynamic organelles that undergo continuous fusion and fission.¹²⁰ During this process, dysfunctional mitochondria are separated and degraded by mitophagy, which acts as a quality control mechanism.¹²¹ Palmitate induces mitochondrial depolarization, morphodynamic fragmentation and impaired ATP synthesis.³⁰ Furthermore, palmitate suppresses autophagic activity, which may increase the proportion of dysfunctional mitochondria. Defective fission allows mitochondria to become more elongated but also more susceptible to glucolipotoxicity.^{121,122} The deterioration of mitochondrial function induces PT pore opening followed by caspase activation and apoptosis. The major known stimuli for PT pore opening are oxidative stress and matrix Ca²⁺ overload, both of which are observed during palmitate overload (Figure 2). Mitochondrial antioxidants effectively protect from palmitate-induced ER Ca²⁺ depletion, IP₃ generation, ER stress and cell death.³⁰ These findings demonstrate the important role of mitochondrial ROS in the palmitate-induced vicious cycle of calcium dysregulation and apoptosis.

FUNCTIONAL CONSEQUENCES OF LIPOTOXICITY AND IMPLICATIONS

Pancreatic beta cell failure and diabetes

During the glucose stimulation of pancreatic β -cells, insulin synthesis represents more than half of total protein synthesis in this highly specialized cell type. This high synthesis rate of insulin is further exaggerated in the context of insulin resistance, when proinsulin production is approximately 1 000 000 molecules per minute.¹²³ Therefore, ER function in β -cells is prone to be overloaded in individuals on a high-calorie diet with limited physical activity. Saturated FFAs exert extra stress on β -cells due to the induction of ROS production. In an attempt to overcome this stress, β -cells upregulate the expression of chaperone proteins and reduce the ER workload as part of the ER stress response. Once a threshold of ER stress has been reached, palmitate may shift the β -cell response from physiologic adaptation to a pro-apoptotic program.¹²⁴

ER stress in β -cells is a critical step in the pathogenesis of type 2 diabetes (Figure 3). Both high glucose and lipid stimulation produce mitochondrial ROS synergistically. Compared to other cell types, β -cells are highly susceptible to oxidative stress. High glucose and/or palmitate have been reported to decrease SERCA2b expression and ER Ca²⁺ level in β -cells.⁹² Inflammatory cytokines, acting as pathogenic molecules in type 1 diabetes, also attenuate SERCA2b expression in β -cells.¹²⁵ Therefore, ER stress caused by insufficient ER Ca²⁺ content may be an important factor in the development of diabetes. In addition, the deletion or inactivation of WFS1,

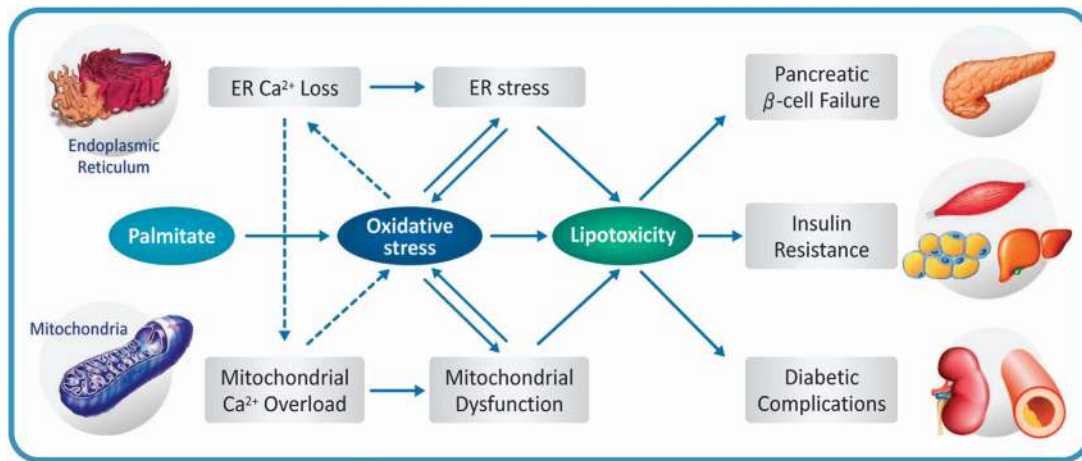


Figure 3 Proposed mechanism of lipotoxicity in type 2 diabetes. Oxidative stress and calcium dysregulation form a vicious cycle that disturbs critical organelle function. Lipotoxicity resulting from ER stress and mitochondrial dysfunction contributes to pancreatic β -cell failure, insulin resistance in target tissues and diabetic complications.

which is mutated in Wolfram syndrome, results in reduced ER Ca²⁺ content and increases ER stress in β -cells.^{92,126} Genome studies revealed a link between WFS1 polymorphism and a high risk of type 2 diabetes,¹²⁷ which may be due to the reported ER stress in β -cells. Palmitate, but not oleate, has been shown to trigger NF- κ B activation and ER stress, which may be one mechanism to induce interleukin 1 β (IL-1 β) and downstream chemokines and cytokines, culminating in mild inflammation in human islets, although this does not directly cause β -cell dysfunction and apoptosis.¹²⁸

Mitochondrial function in β -cells is particularly important because glucose/lipid/amino acid metabolism and insulin secretion depend on mitochondrial function.¹²⁹ It has been demonstrated that mitochondrial morphodynamics protect β -cells from lipotoxicity.¹³⁰ The inhibition of mitochondrial fission and/or defective mitophagy augments sensitivity to glucolipotoxicity.¹²² Mitochondrial Ca²⁺ is a crucial regulator of mitochondrial energy metabolism,¹⁰⁸ as mentioned earlier. Therefore, Ca²⁺ transport from the ER to the mitochondria can affect mitochondrial metabolism as well as β -cell death. The pathogenic role of the ER-mitochondrial Ca²⁺ connection in mitochondrial dysfunction and β -cell failure by palmitate deserves further investigation.

Insulin resistance in target tissues

It is well-known that palmitate induces insulin resistance by disrupting intracellular insulin signaling in diverse cell types such as hepatocytes, cardiac and skeletal muscle cells, adipocytes, podocytes, hypothalamic neurons, and pancreatic α -cells.^{42,131–136} Palmitate exposure activates JNK, which phosphorylates IRS-1 on serine³⁰⁷ and decreases Akt phosphorylation, leading to the impairment of downstream signaling. Intriguingly, neuronal cells are more prone to the cytotoxic effects of palmitate. Compared to other cell types, neuronal cells are sensitive to lower doses and shorter exposure time.¹³⁵ Oleate, again, prevents palmitate-induced insulin resistance in many cases.^{137–139}

Palmitate-induced oxidative stress is the main mechanism disrupting insulin signaling (Figure 3). As discussed above, ROS are derived from multiple sources: mitochondrial ETC, DAG-PKC-NOX and CHOP-ERO1 α . ROS can activate not only JNK but also other serine kinases, such as p38 MAPK, GSK-3 β and IKK β in skeletal muscle.¹⁴⁰ In HepG2 cells treated with palmitate, p38 MAPK and JNK activities are significantly attenuated by siRNA-mediated NOX3 silencing.⁴² However, in another hepatic cell line, ROS-induced JNK activation was not completely reversed, even when efficiently suppressing ROS levels using antioxidants.⁴⁵ The findings suggest that other mechanisms are also involved in palmitate-induced insulin resistance. One possible explanation is the intracellular accumulation of ceramide, which may activate JNK via mixed lineage kinase-3.^{141,142}

Ca²⁺ is another modulator of insulin signaling, the molecular mechanisms of which are still poorly understood.¹⁴³ Ca²⁺/calmodulin was suggested to have an important role in the insulin-mediated translocation and exocytosis of glucose transporter type 4 (GLUT4) vesicles in 3T3-L1 adipocytes. A more recent study found that the Ca²⁺ chelator BAPTA¹⁴⁴ operates through the depolarization of microtubules rather than Ca²⁺ chelation.¹⁴⁵ In L6 myotubes, ER Ca²⁺ release through both ryanodine receptor 1 (RYR1) and IP₃R promotes insulin-dependent GLUT4 trafficking to the plasma membrane.¹⁴⁶ Palmitate impairs mitochondrial calcium retention capacity and impairs insulin-stimulated GLUT4 translocation in L6 myotubes, which was fully restored by adding an inhibitor of PT pore opening.¹⁴⁷ Finally, several studies have suggested that either enlarged or insufficient MAMs fail to maintain normal ER-mitochondrial Ca²⁺ homeostasis. Altered MAM structures may, therefore, indirectly affect the translocation and fusion of GLUT4 vesicles with the plasma membrane.¹⁴⁸ Does palmitate-induced ER-mitochondrial Ca²⁺ dysregulation affect GLUT4 trafficking in a ROS-independent manner? Does palmitate affect insulin-dependent and/or contraction-dependent GLUT4 translocation

in muscle? More studies are needed to address such potential Ca²⁺-mediated mechanisms of lipotoxicity.

Diabetic complications

Chronic diabetic complications have traditionally been attributed to long-term exposure to high glucose. The four classical pathways of hyperglycemia-induced complications include (1) increased polyol pathway flux, (2) increased intracellular formation of advanced glycation end products (AGE), (3) the activation of PKC and (4) the stimulation of the hexosamine pathway. Those pathways are connected by the fact that intracellular high glucose induces elevated mitochondrial ROS production, which leads to a decrease in GAPDH activity. As a result, upstream glycolytic metabolites are diverted into the pathogenic pathways described above.¹⁴⁹ In addition, high glucose augments the expression and activity of members of the NOX family.^{150–152} A detailed description of this crosstalk between NOX and mitochondrial ROS generation has been described elsewhere.^{55,153}

Intriguingly, accumulating evidence supports a synergistic effect between palmitate and high glucose leading to diabetic complications. Such findings have led to the concept of glucolipotoxicity. Oxidative stress may be a common mechanism explaining the harmful synergistic effects of the two nutrients. In bovine and human retinal endothelial cells, NOX2-derived ROS overproduction was significantly higher when the cells were exposed to palmitate and high glucose rather than high glucose alone. Consequently, mitochondrial DNA damage is observed as early as 48 h when bovine retinal cells are exposed to palmitate and high glucose. Similar mtDNA damage was only observed after 96 h when high glucose was added alone.¹⁵⁴ The separate exposure of HUVEC cells to either palmitate or high glucose increases ROS production, but the highest ROS levels were observed upon treatment with both.¹⁵⁵ These experiments clearly demonstrate that glucolipotoxicity, which was originally proposed to affect β -cells, may be similarly harmful to other tissues.

In diabetic nephropathy, functional and structural alterations in podocytes accompany disease progression. In this cell type, palmitate reduces tyrosine phosphorylation following insulin stimulation.¹⁵⁶ This defect downstream of insulin receptor signaling also impairs GLUT4 translocation in podocytes. Palmitate-induced intracellular calcium dysregulation also participates in diabetic nephropathy. In podocytes, elevated cytosolic Ca²⁺ concentrations induce actin remodeling, which increases albumin permeability. These structural alterations in podocytes also have a critical role in the pathogenesis of proteinuric glomerular disease.³⁰

Furthermore, elevated palmitate may also exert harmful effects in periodontitis linked to type 2 diabetes. In mice fed a high-fat diet, which serve as a model of type 2 diabetes, CD36 is overexpressed in gingival fibroblasts. In human gingival fibroblasts, palmitate also provokes mRNA expression of pro-inflammatory cytokines and chemokines, as well as IL-6, IL-8 and CXCL1.¹⁵⁷ This evidence supports the hypothesis that palmitate exposure may worsen diabetic complications.

CONCLUSION

The accumulation of palmitate and derived metabolites, e.g., DAG, induces oxidative stress and ER Ca²⁺ depletion, leading to ER stress and mitochondrial dysfunction. Excessive ER Ca²⁺ release and mitochondrial Ca²⁺ overload further amplify oxidative stress. This close interaction between oxidative stress and Ca²⁺ dysregulation results in a vicious cycle of increasingly impaired cell function and death. The activation of stores-operated Ca²⁺ entry may chronically disturb cytosolic and organellar Ca²⁺ homeostasis; this hypothesis will require further investigation. The disruption of Ca²⁺ regulation by oxidative stress also contributes to insulin resistance. These hypotheses provide an integrated mechanistic view of lipotoxicity, which has pivotal roles during the progress of diabetes and its complications. In this review, we have suggested future therapeutic approaches to type 2 diabetes via interference with the basic molecular mechanisms overstimulated during lipotoxicity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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