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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 $Ca^{2+}$ homeostasis. Highly oxidative conditions induced by palmitate trigger aberrant endoplasmic reticulum (ER) $Ca^{2+}$ release and thereby deplete ER $Ca^{2+}$ stores. The resulting ER $Ca^{2+}$ 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 $Ca^{2+}$ release, cytosolic and mitochondrial matrix $Ca^{2+}$ concentrations can also be altered. In addition, plasmalemmal ion channels operated by ER $Ca^{2+}$ depletion mediate persistent $Ca^{2+}$ influx, further impairing cytosolic and mitochondrial $Ca^{2+}$ homeostasis. Mitochondrial $Ca^{2+}$ 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 $Ca^{2+}$-mediated pathogenic changes. These mechanisms show harmful cross-interactions. Endoplasmic reticulum (ER) $Ca^{2+}$ release due to palmitate-induced oxidative stress results in cytosolic and mitochondrial $Ca^{2+}$ 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 $Ca^{2+}$ (SOC) entry triggered by ER $Ca^{2+}$ depletion augments the persistent $Ca^{2+}$ load. The interruption of such vicious cycles of ROS formation and $Ca^{2+}$ 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-cells[13] and podocytes[14] 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, $IP_3$-mediated ER $Ca^{2+}$ 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 $Ca^{2+}$ activates $Ca^{2+}$-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] hepatocytes[42] 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 Cα (PKCα) by palmitate increases ROS production through NOX2 upregulation in cardiomyocytes.[38]

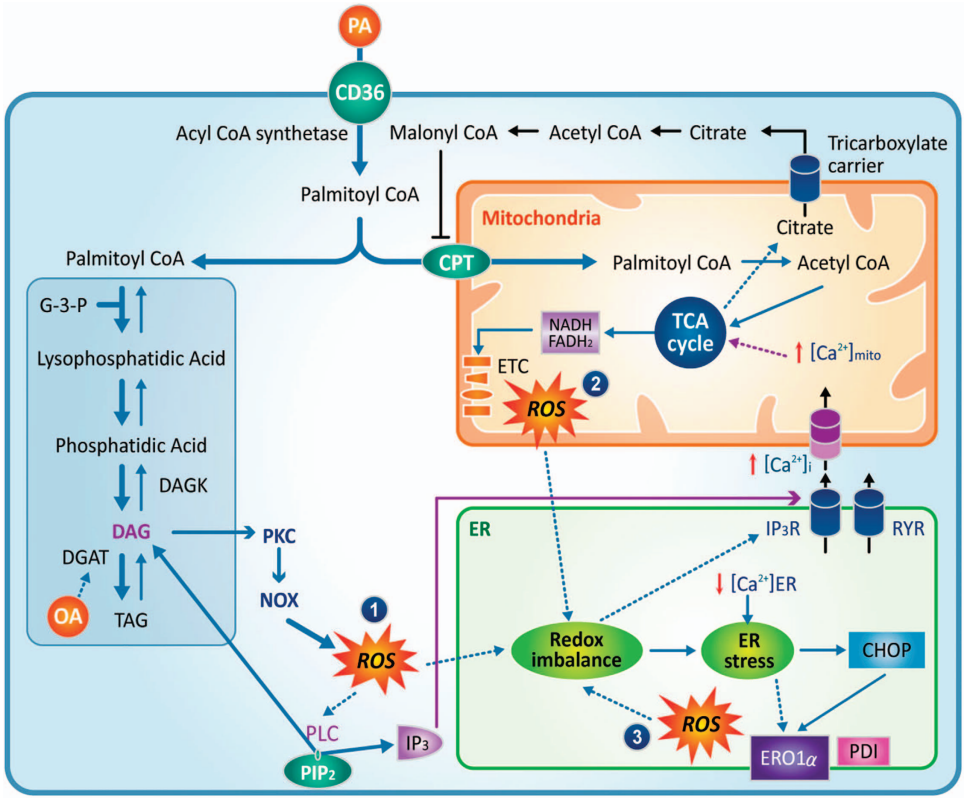


**Figure 1** Palmitate induces ROS overproduction. (1) Increased β-oxidation, (2) DAG-PKC-NOX, (3) CHOP-ERO1α and PDI under ER stress. ROS produced by palmitate triggers PLC activation, ER $Ca^{2+}$ release, ER stress and mitochondrial dysfunction, which, in turn, aggravate ROS generation. CHOP, CCAAT-enhancer-binding protein homologous protein; DAG, diacylglycerol; ERO1α, 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 (ERO1α) and disulfide isomerase during ER stress, as well as ER-mitochondrial $Ca^{2+}$ 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 $H_2O_2$ 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 artery[59] 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 1α (IRE1α), 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 $Ca^{2+}$ 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 $^{3}$H-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, α1 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 eIF2α 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

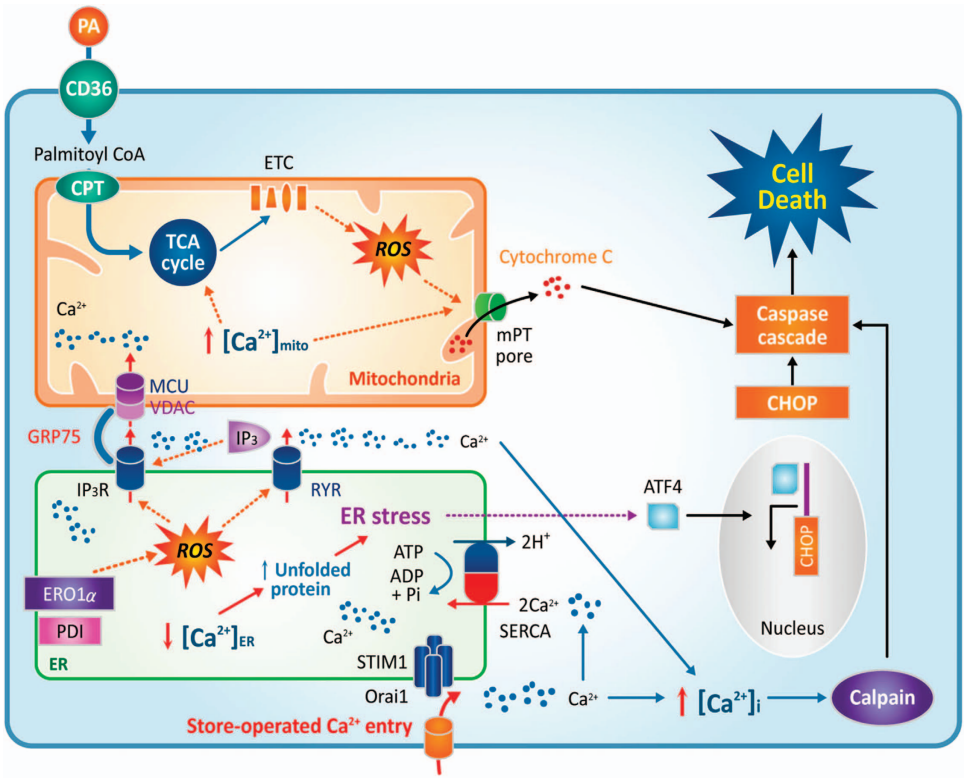


**Figure 2** Palmitate disturbs intracellular $Ca^{2+}$ homeostasis. ROS activate $IP_3R$ and RYR, which release $Ca^{2+}$ from the ER. The deprivation of ER $Ca^{2+}$ leads to ER stress and CHOP upregulation. $Ca^{2+}$ is transported into mitochondria through a specialized structure composed of $IP_3R$, VDAC, MCU and GRP75. Excessive $Ca^{2+}$ in mitochondria leads to cytochrome *c* release. SOC entry triggered by ER $Ca^{2+}$ depletion elicits the persistent influx of $Ca^{2+}$ 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 $Ca^{2+}$ uniporter; ROS, reactive oxygen species; SOC, store-operated $Ca^{2+}$; VDAC, voltage-dependent anion channel.

homologous protein (CHOP)) has protective effects on palmitate-induced apoptosis in insulin-secreting cells,[68,88] podocytes[75] 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 $Ca^{2+}$ concentration is particularly important for protein folding. High levels of $Ca^{2+}$ in the ER lumen ($>400\ \mu M$) are required for interactions among ER chaperones and between chaperones and unfolded proteins.[66] SERCA maintains high ER $Ca^{2+}$ concentrations. A chronic reduction of ER $Ca^{2+}$ 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 $Ca^{2+}$ stores. Palmitate-induced ER stress is also associated with a sustained reduction of the ER $Ca^{2+}$ pool, which has been demonstrated directly through cytosolic and ER $Ca^{2+}$ measurements.[30,68,88,91] ER $Ca^{2+}$ 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 $IP_3$ receptors ($IP_3R$) and stimulates ER $Ca^{2+}$ release.[95] Consequent ER $Ca^{2+}$ 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 $IP_3R$ activation and ER $Ca^{2+}$ release (Figure 1). Blocking this vicious cycle between ER ROS formation and ER $Ca^{2+}$ release could be a pertinent therapeutic strategy. In support of this approach, we observed that palmitate-induced ER $Ca^{2+}$ loss was prevented by both ROS scavengers or the inhibition of $IP_3$ generation.[30]

It is noteworthy that $H_2O_2$-mediated oxidative stress can activate PLCγ and generate $IP_3$ 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 $H_2O_2$. Pretreatment with a PLC inhibitor attenuated palmitate-induced ER $Ca^{2+}$ loss, suggesting that $IP_3$ generation from phosphatidylinositol 4,5-bisphosphate ($PIP_2$) contributes to ER $Ca^{2+}$ release via the $IP_3R$.[30] In addition, DAG, the other signaling molecule produced by PLC activity, may also participate in palmitate-dependent ER $Ca^{2+}$ loss. This hypothesis was supported by experimental evidence showing that palmitate-induced ER $Ca^{2+}$ 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 $Ca^{2+}$ loss. The inhibition of PKC blunted the effect of palmitate on ER $Ca^{2+}$, suggesting a critical pathogenic role for DAG-mediated PKC activation.[30] We propose a synergistic stimulation of $Ca^{2+}$ release from the ER by $IP_3$ and DAG, although further detailed studies are required to substantiate our working model.

## PALMITATE DISTURBS INTRACELLULAR CALCIUM HOMEOSTASIS

Plasma membrane $Ca^{2+}$ ATPase (PMCA) and SERCA establish 1000- to 10 000-fold change gradients of $Ca^{2+}$ concentrations across the plasma membrane and the ER membrane.[90] Therefore, inappropriate and uncontrolled cytosolic $Ca^{2+}$ increases that result from $Ca^{2+}$ influx from the extracellular space or release from the ER are a burden for the cell as it tries to maintain intracellular $Ca^{2+}$ homeostasis. $Ca^{2+}$ stress may initiate pathogenic processes such as calpain-mediated cell death. In β-cells, for instance, it was observed that palmitate-induced ER $Ca^{2+}$ release activates the calcium-dependent pro-apoptotic protease calpain-2.[92]

$Ca^{2+}$ release from the ER participates in cell death mechanisms. The luminal ER $Ca^{2+}$ 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 $IP_3$ receptor, the voltage-dependent anion channel and the mitochondrial $Ca^{2+}$ uniporter (MCU). Additional adaptor proteins, including GRP75, are also required to establish high capacity $Ca^{2+}$ 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 $Ca^{2+}$ depletion

and mitochondrial $Ca^{2+}$ overload, although this hypothesis requires further experimentation.[104]

The activation of $Ca^{2+}$ signals via PLC-mediated $IP_3$ generation depletes ER $Ca^{2+}$ 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 $Ca^{2+}$ reservoir. In the ER membrane, the stromal interaction molecule (STIM), a transmembrane protein with luminal EF hands, senses ER $Ca^{2+}$ levels. A decrease in ER $Ca^{2+}$ 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 $Ca^{2+}$ channel. Orai1 mediates SOC entry until ER $Ca^{2+}$ stores are refilled, at which point STIM oligomers again become dispersed. Notably, palmitate-treated cells maintain STIM1 oligomerization, signifying that ER $Ca^{2+}$ release and depletion of stores persist.[30] Upon extracellular $Ca^{2+}$ addition, palmitate-treated cells show strong and sustained increases in cytosolic $Ca^{2+}$, whereas there is a negligible influence on $Ca^{2+}$ influx in control cells (Figure 2).[30] This evidence suggests that ER $Ca^{2+}$ depletion by palmitate induces sustained SOC entry, which may raise cytosolic and mitochondrial $Ca^{2+}$ to an intolerable level. Low extracellular $Ca^{2+}$ conditions protect against palmitate-induced cytotoxicity, suggesting that SOC contributes to the harmful effects of palmitate.[105] It should be noted that $Ca^{2+}$ 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, $Ca^{2+}$ homeostasis and the integration of apoptotic signals.[106,107] $Ca^{2+}$ in the mitochondrial matrix and extramitochondrial locations modulates mitochondrial functions, including intermediary metabolism and ATP synthesis. Mitochondrial $Ca^{2+}$ activates pyruvate dehydrogenase, Krebs cycle activity, mitochondrial transporters, and ATP synthase.[108–110] MCU is the main molecule responsible for mitochondrial $Ca^{2+}$ 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 $Ca^{2+}$ arrest the movement of mitochondria, allowing the organelle to efficiently take up and sequester $Ca^{2+}$ into its matrix to stimulate mitochondrial energetics.[115] Excess mitochondrial $Ca^{2+}$ 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 $Ca^{2+}$ overload, both of which are observed during palmitate overload (Figure 2). Mitochondrial antioxidants effectively protect from palmitate-induced ER $Ca^{2+}$ depletion, $IP_3$ 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 $Ca^{2+}$ 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 $Ca^{2+}$ content may be an important factor in the development of diabetes. In addition, the deletion or inactivation of WFS1,

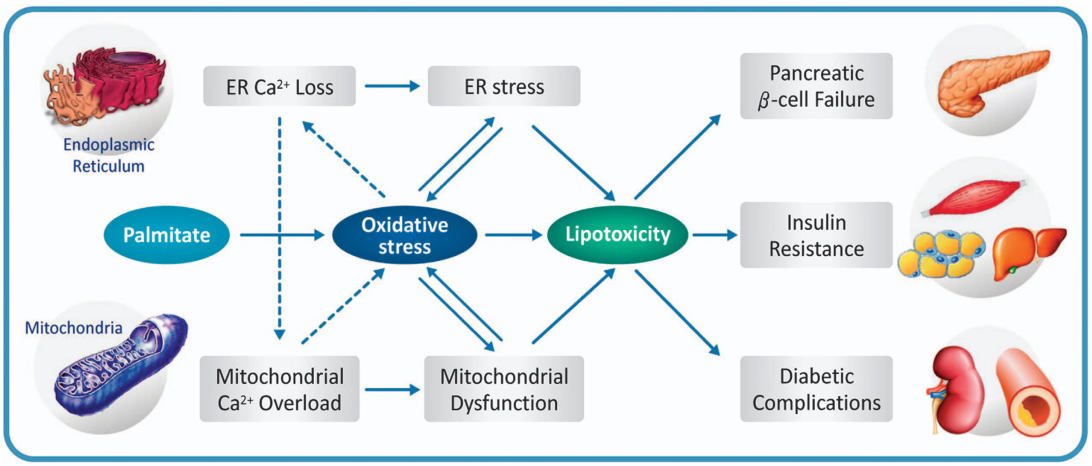


**Figure 3** Proposed mechanism of lipotoxicity in type 2 diabetes. Oxidative stress and calcium dysregulation form a vicious cycle that disturbs critical organelle function. Lipotoxicity resulting from ER stress and mitochondrial dysfunction contributes to pancreatic β-cell failure, insulin resistance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

which is mutated in Wolfram syndrome, results in reduced ER $Ca^{2+}$ 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-κB activation and ER stress, which may be one mechanism to induce interleukin 1β (IL-1β) 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 $Ca^{2+}$ is a crucial regulator of mitochondrial energy metabolism,[108] as mentioned earlier. Therefore, $Ca^{2+}$ transport from the ER to the mitochondria can affect mitochondrial metabolism as well as β-cell death. The pathogenic role of the ER-mitochondrial $Ca^{2+}$ 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 serine[307] 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-ERO1α. ROS can activate not only JNK but also other serine kinases, such as p38 MAPK, GSK-3β and IKKβ 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]

$Ca^{2+}$ is another modulator of insulin signaling, the molecular mechanisms of which are still poorly understood.[143] $Ca^{2+}$/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 $Ca^{2+}$ chelator BAPTA[144] operates through the depolarization of microtubules rather than $Ca^{2+}$ chelation.[145] In L6 myotubes, ER $Ca^{2+}$ release through both ryanodine receptor 1 (RYR1) and $IP_3R$ 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 $Ca^{2+}$ 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 $Ca^{2+}$ 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 $Ca^{2+}$-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 $Ca^{2+}$ 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 $Ca^{2+}$ depletion, leading to ER stress and mitochondrial dysfunction. Excessive ER $Ca^{2+}$ release and mitochondrial $Ca^{2+}$ overload further amplify oxidative stress. This close interaction between oxidative stress and $Ca^{2+}$ dysregulation results in a vicious cycle of increasingly impaired cell function and death. The activation of stores-operated $Ca^{2+}$ entry may chronically disturb cytosolic and organellar $Ca^{2+}$ homeostasis; this hypothesis will require further investigation. The disruption of $Ca^{2+}$ 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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