Trends in Endocrinology & Metabolism

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REVIEWS

Review

Calcium Signaling As a Therapeutic Target for Liver Steatosis

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Hepatic steatosis, the first step in nonalcoholic fatty liver disease (NAFLD), can arise from various pathophysiological conditions. While lipid metabolism in the liver is normally balanced such that there is no excessive lipid accumulation, when this homeostasis is disrupted lipid droplets (LDs) accumulate in hepatocytes resulting in cellular toxicity. The mechanisms underlying this accumulation and the subsequent hepatocellular damage are multifactorial and poorly understood, with the result that there are no currently approved treatments for NAFLD. Impaired calcium signaling has recently been identified as a cause of increased endoplasmic reticulum (ER) stress contributing to hepatic lipid accumulation. This review highlights new findings on the role of impaired Ca^{2+} signaling in the development of steatosis and discusses potential new approaches to NAFLD treatment based on these new insights.

Highlights

Insulin resistance in hepatocytes, endoplasmic reticulum stress, and lipid storage capacities are all important in the development of hepatic steatosis.

Store-operated Ca^{2+} entry in liver cells has important regulatory properties in liver lipid synthesis.

Calcium deficiency caused by reduced store-operated Ca^{2+} entry (SOCE) function may be a new crucial player in the development of liver steatosis.

Reduced SOCE function increases the rate of accumulation of intracellular lipids, raising the possibility that reduced SOCE function may contribute to nonalcoholic steatohepatitis and insulin resistance.

Therapies able to reactivate SOCE and/or reduce protein kinase C action and/or stimulate SERCA activity may be useful in treatment of hepatic steatosis, insulin resistance, and type 2 diabetes.

Hepatic Steatosis, Nonalcoholic Steatohepatitis (NASH) and Store-Operated Ca^{2+} Entry (SOCE)

Hepatic **steatosis** (see Glossary) is a common liver disorder affecting about 17–40% of the population in Western countries and 2–4% worldwide [1–5]. Accumulation of excess LDs in **hepatocytes** can lead to hepatic steatosis, which may progress to **nonalcoholic steatohepatitis (NASH)**, hepatic **insulin resistance**, and **type 2 diabetes (T2D)**. The exact mechanisms by which LDs form in hepatocytes in **NAFLD** is still largely unknown. Regulation of liver metabolism is physiologically dependent on hormone-mediated release of Ca^{2+} from ER stores from hepatocytes and subsequent refilling of these stores by Ca^{2+} entry through store-operated Ca^{2+} channels (SOCs). Recent evidence suggests that steatosis leads to impaired intracellular Ca^{2+} regulation and ER function resulting in a vicious cycle of further LD formation and accumulation [6–9]. Hepatic steatosis is thereby a common road with multiple on-ramps [4,5]. This review critically discusses the role of SOCE (store-operated Ca^{2+} entry) in liver steatosis and the effects of steatosis on **intracellular Ca^{2+} homeostasis** and lipid synthesis. In addition, we apply this new knowledge of liver Ca^{2+} imbalance to understanding of potential treatments for hepatic steatosis and insulin resistance.

Hormonal Regulation of Hepatic Metabolism Involves Ca^{2+} Signaling

Hepatocyte activity is regulated by hormones and growth factors, which use changes in Ca^{2+} concentrations as intracellular signals [10,11]. Regulation of liver metabolism involves many different hormones, with **insulin** and glucagon playing major roles. Epinephrine, vasopressin, and norepinephrine also play important roles in the regulation of liver metabolism [10,11]. Hormonal regulation of hepatic metabolism involves various intracellular messengers, including cAMP, cGMP, Ca^{2+}, and protein kinase C (PKC) [9–11].

Insulin, glucagon, epinephrine, and other hormones regulate metabolic responses in the liver via changes in cytoplasmic Ca^{2+} concentration. In single hepatocytes, hormone-initiated

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increases in cytoplasmic Ca^{2+} are encoded as the frequency of Ca^{2+} oscillations, which regulate carbohydrate and lipid metabolism [11]. Ca^{2+}-calmodulin-dependent kinase II plays an important role in the regulation of glycogen and glucose synthesis [12]. While the citric acid cycle and ATP synthesis in hepatocytes are regulated by the concentration of Ca^{2+} in the mitochondrial matrix, the concentration of Ca^{2+} in the ER regulates the metabolism of xenobiotic compounds, as well as protein and lipid synthesis [5,8,11].

In hepatocytes, cytoplasmic Ca^{2+} is an important regulator of glucose and lipid metabolism, bile secretion, mitochondrial activity, cell motion, cell volume, cell growth, cell survival, and **apoptosis** [7,10]. In normal cells, multiple components are dedicated to maintaining the optimal cytoplasmic Ca^{2+} concentration [13]. Hormone-mediated increases in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) are mediated by phospholipase C activation [11], inositol 1,4,5-trisphosphate (IP_3) generation, and the release of ER Ca^{2+} stores. The depleted ER Ca^{2+} is subsequently replenished with Ca^{2+} inflow through the activation of SOCE pathways [13,14]. For details of SOC modulators, STIM/Orai-interacting proteins, and the regulation of channel activity (by pharmacological inhibitors), readers are referred to an excellent review [13].

Hepatic SOC channels principally comprise STIM1 and Orai1 proteins [8]. STIM1, acting as a Ca^{2+} sensor, is located in the ER membrane during the resting condition of the cells. STIM1 can sense a decrease in the concentration of ER Ca^{2+} and moves towards the plasma membrane where it binds to Orai1. Subsequently, the Orai1 pore is activated allowing Ca^{2+} entry from the extracellular space [15,16]. While intracellular Ca^{2+} in the range of 0.1–2 μM is a vital messenger and regulator of cellular pathways, decreased or excess intracellular Ca^{2+} is toxic to cells [5,9,11,17,18].

The ER normally maintains intracellular calcium homeostasis. Under physiological conditions in hepatocytes, the ER releases Ca^{2+} from its lumen to the cytoplasm predominantly by IP_3R-mediated pathways. Conversely, Ca^{2+} is moved from the cytoplasm into the hepatocyte ER lumen by calcium-ATPase pumps including sarco(endo)plasmic reticulum ($Ca^{2+} + Mg^{2+}$) ATPase (SERCA), thereby maintaining a dynamic balance of Ca^{2+} [19,20]. The function of calcium-ATPase pumps is vital to calcium homeostasis and thereby normal ER functions such as protein folding, modification, and trafficking [19]. Several lines of evidence suggest that excessive lipid accumulation in hepatocytes inhibits SOCE [16] and reduces ER Ca^{2+} content through excess PKC phosphorylation of Orai1 [16], through Cisd2 **haploinsufficiency** [5], or through reduced SERCA2b levels [21,22]. This aberrant calcium homeostasis has been implicated in ER stress-related metabolic pathologies such as hepatic steatosis, **obesity**, and T2D [16,18,23].

Nonalcoholic Steatosis, Steatohepatitis (NASH), and Insulin Resistance

Hepatic steatosis is associated with obesity and the **metabolic syndrome**. In some patients, simple steatosis develops into liver inflammation and NASH [3,17], which in turn may lead to hepatocellular carcinoma [4,5,9]. Hepatic lipid accumulation may reflect increased liver lipid influx together with increased liver **lipogenesis** [7,8]. Faulty mitochondrial oxidation and lipid export may also contribute to hepatic lipid accumulation [7]. Hepatic steatosis may lead to hepatic insulin resistance and once this develops, this causes impaired insulin-mediated suppression of hepatic glucose production, which potentially could lead to fasting **hyperglycemia** and may thereby ultimately contribute to the development of T2D [24].

How Excessive LD Accumulation Alters Liver Function

Lipid accumulation in hepatocytes is associated with mitochondrial dysfunction and the generation of **reactive oxygen species (ROS)** [25]. ROS, in turn, oxidize fat droplets to release lipid peroxidation products that are toxic to hepatocytes [25]. ROS may also induce the

Glossary

Allosteric: allosteric activation is the activation/regulation of an enzyme by the binding of an effector molecule at a site other than the enzyme's active site.
Antisense oligonucleotides: short, synthetic, single-stranded oligodeoxynucleotides that can alter RNA and reduce, restore, or modify protein expression through several mechanisms.
Apoptosis: the programmed death of cells that occurs as a normal and controlled part of an organism's development or growth.
Gluconeogenesis: the synthesis of glucose from non-sugar precursors, such as lactate, pyruvate, and the carbon skeleton of glucogenic amino acids.
Haploinsufficiency: describes the situation where having only a single functioning copy of a gene is not enough for normal function, so that loss-of-function mutations cause a dominant phenotype.
Hepatocytes: the chief functional cells of the liver; perform a number of metabolic, endocrine, and secretory functions. Roughly 80% of the mass of the liver is contributed by hepatocytes.
Hyperglycemia: elevated amount of sugar (glucose) in the blood beyond normal, often associated with diabetes mellitus.
Insulin: a dimeric peptide hormone (51 amino acids) comprising an A chain and a B chain linked by disulfide bonds. It is produced by β cells of pancreatic islets.
Insulin resistance: decreased cellular response to insulin.
Intracellular Ca^{2+} homeostasis: regulation of the intracellular concentration of calcium ions in cells.
Lipogenesis: a process of fatty acid and triglyceride synthesis from glucose or other substrates.
Metabolic syndrome: a disorder of energy utilization and storage defined by the presence of at least three of the five following medical conditions: high blood pressure, high blood glucose, abdominal obesity, high serum triglycerides, and low high-density lipoprotein (HDL) levels.
Microsomal: related to microsomes. A microsome is a fragment of ER and attached ribosomes obtained by

unfolded protein response and ER stress and impair lipid, protein, and cholesterol synthesis in a vicious deleterious cycle [5,8,20] (Figure 1A).

The ER stress response can be caused by the perturbation of any of the homeostatic functions (e.g., intracellular Ca^{2+} storage, lipid synthesis, protein folding) of the ER [5]. Hence, decreased ER luminal Ca^{2+} concentration in steatotic liver cells of obese mice can lead to the ER stress response [21,22]. Prolonged activation of the ER stress response has been shown to lead to insulin resistance and diabetes in animal models of obesity and obese human patients [12,18]. Continuous exposure to excess nutrients, such as fatty acids and glucose, places additional load on the ER adaptive stress responses [26]. When compensatory mechanisms fail, this may increase the risk of development of chronic metabolic diseases such as T2D [6,18,26].

Increased plasma insulin results in suppression of hepatic glucose production [27]. Visceral adiposity may cause free fatty acids to flux to the liver via the portal vein. This leads to the accumulation of LDs in the liver, resulting in steatosis and hepatic insulin resistance [27]. While insulin resistance may occur in a range of target tissues, it has a severe impact on the liver [27]. Hepatic insulin resistance may be mediated by reduced insulin-stimulated IRS-2 tyrosine phosphorylation by insulin receptor kinase [28]. In a mouse model of metabolic syndrome, the development of steatohepatitis, an upstream process of insulin resistance, was associated with impaired hepatic fatty acid disposal pathways and T2D [29,30]. Hepatic LDs can activate PKC, which in turn may inhibit insulin receptor kinase function. Impaired insulin receptor kinase function further impairs tyrosine phosphorylation of IRS-1, leading to increased **gluconeogenesis** via the MEK pathway [31,32]. LDs may also impair the effects of insulin-like growth factor-I (IGF-I) [32], leading to insulin resistance.

The synthesis of chaperone proteins can be increased to compensate for impaired protein folding and ER stress [18,33,34]. The activation of several isoforms of PKC may reduce ER Ca^{2+} content [16] leading to insulin resistance [35]. Although steatosis can also be associated with hepatic glucagon resistance, steatosis is predominantly associated with hepatic insulin resistance. Figure 1A shows schematically the stepwise development of insulin resistance in steatotic hepatocytes. Hepatic steatosis has been shown to affect the levels of many different hormones [16].

PKC activation driven by lipid accumulation (Figure 1A) is likely to play a role in the development of hepatic insulin resistance [36,37]. PKCβ, PKCδ, and PKCε are activated in the steatotic liver [1,31,35,37]. Knockdown of PKCε expression in rat liver prevented hepatic insulin resistance, confirming its key role [36]. Production of diacylglycerols (DAGs) through the glycerol 3-phosphate pathway represents the lipogenesis route in the synthesis of triacylglycerols (TAGs) and phospholipids, with the level of DAGs in the hepatocyte cytosol correlating with PKC activation and insulin resistance [1]. Moreover, PKC activation was shown to be linked to reduced intracellular and ER Ca^{2+} storage capacity in HEK cells [38] and with development of ER stress in human vascular smooth muscle cells [39].

Ca^{2+} Signaling Is Altered in Steatotic Hepatocytes

The high concentration of Ca^{2+} within the ER in hepatocytes is maintained by the SERCA2b protein [5]. Park and colleagues showed that there is a significant reduction in SERCA2b protein and mRNA levels in the liver ER **microsomal** fractions of obese versus lean control mice [21]. Steatosis has also been shown to be associated with impaired activity of SERCA2b at the ER membrane [22]. The activity of SERCA2 is strongly influenced by the nature and composition of the hydrophobic lipid membrane of the ER. The ideal environment for the SERCA2 transporter

the centrifugation of homogenized cells.

Nonalcoholic fatty liver disease (NAFLD): an umbrella term for a range of liver conditions in which fat accumulates in the liver. In general, hepatic steatosis is considered as the first step in NAFLD. NASH is a type of NAFLD.

Nonalcoholic steatohepatitis (NASH): inflammation and liver cell damage along with fat in the liver.

Obesity: a chronic multifactorial disease in which excess accumulation of fat results in adverse health consequences. Clinically, obesity in adults is defined as body mass index >30 kg/m^2.

Reactive oxygen species (ROS): highly reactive chemical compounds that contain oxygen. They are generated by diverse metabolic processes. At low concentrations ROS act as signaling molecules, but at higher concentrations they cause macromolecular damage.

Steatosis: process describing the abnormal accumulation of lipids within a cell. In general, hepatic steatosis is considered the first step in NAFLD.

Store-operated Ca^{2+} (SOC) channel: a plasma membrane Ca^{2+} channel activated by a decrease in Ca^{2+} in the ER; acts to restore the level of calcium in the ER. The entry of Ca^{2+} into the cell is termed store-operated Ca^{2+} entry (SOCE). SOC channels are defined by the fact that they are activated by the decrease in Ca^{2+} in the ER.

Type 2 diabetes (T2D): a chronic condition in which the body fails to properly use and store glucose.

Unfolded protein response: the ER responds to the burden of unfolded proteins in its lumen (ER stress) by activating several intracellular signaling pathways, collectively termed the unfolded protein response.

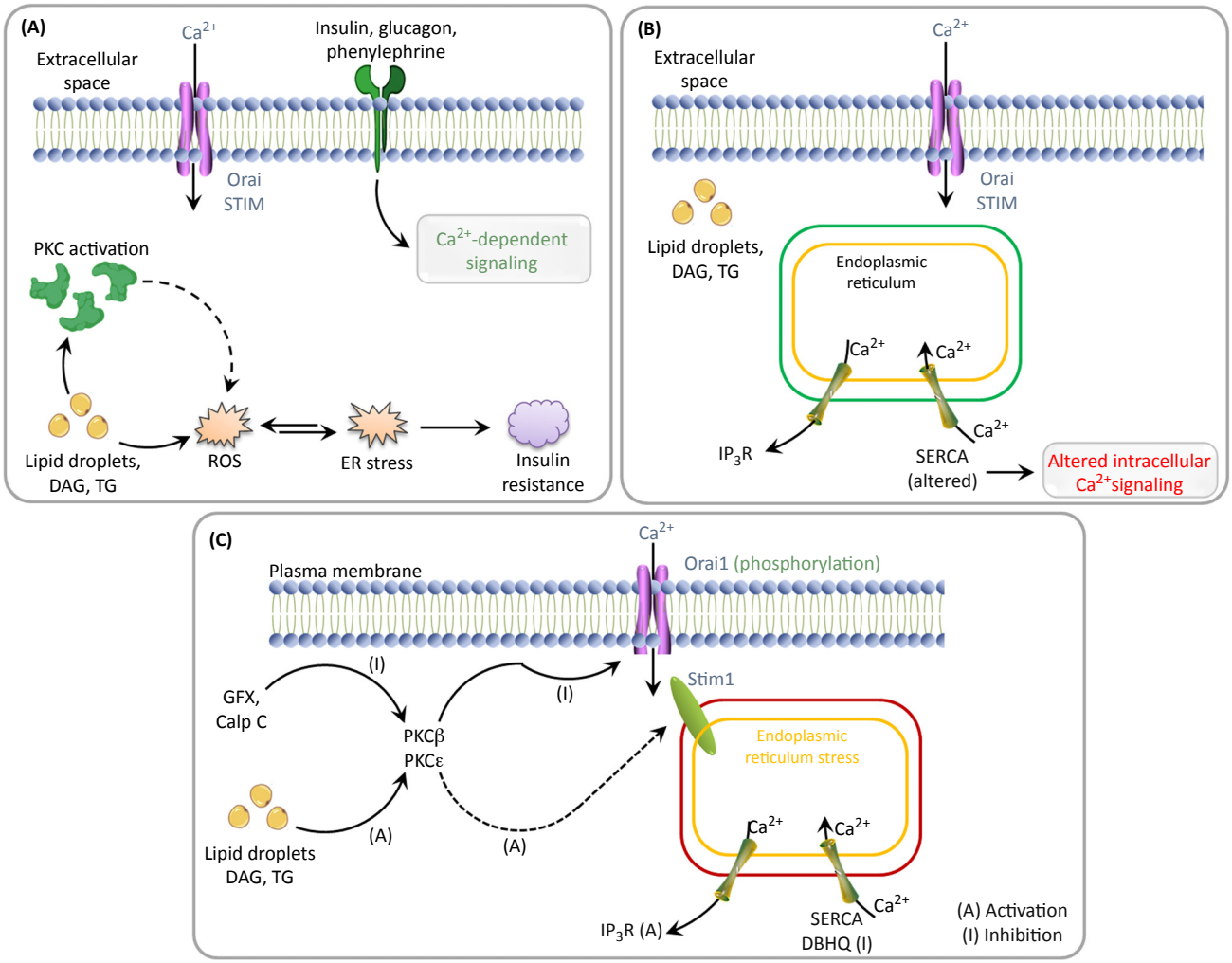

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Figure 1. Mechanisms by Which Excessive Lipid Droplet Accumulation Alters Hepatocyte Ca^{2+} Signaling. (A) Accumulation of lipid droplets and diacylglycerols (DAGs) in hepatocytes may be associated with the generation of reactive oxygen species (ROS), which in turn may cause an ER stress response that ultimately leads to the development of insulin resistance [28]. Lipid accumulation in hepatocytes is also responsible for the activation of various protein kinase C (PKC) isoforms [28]. The single broken arrow denotes that the pathway is not clearly known (in the case of hepatocytes). (B) Expression of the sarco(endo)plasmic reticulum (Ca^{2+} + Mg^{2+}) ATPase 2b (SERCA2b) protein can be altered in steatosis resulting in abnormal Ca^{2+} signaling. Impaired SERCA2b activity/expression due to steatosis may lead to reduced Ca^{2+} in the endoplasmic reticulum (ER) [21,22,26]. TG, triacylglycerol; IP_3R, inositol 1,4,5-trisphosphate receptor; STIM, stromal interaction molecule. (C) A schematic representation of the proposed mechanism for the inhibition of store-operated Ca^{2+} entry (SOCE) in steatotic liver cells. It is proposed that DAG and/or lipid droplets can activate PKC, which in turn leads to PKC-mediated phosphorylation (inhibition) of Orai1, causing inhibition of SOCE in steatotic liver cells. Adapted from [36,74].

protein is a highly fluid membrane lacking in cholesterol [40]. The accumulation of cholesterol during steatosis is associated with marked Ca^{2+} depletion from ER stores, resulting in the induction of the unfolded protein response and cellular apoptosis [41]. It has recently been shown that decreased activity or expression of SERCA2b in the hepatocyte ER in obese mice is linked to a reduction in ER luminal Ca^{2+} concentration [5,21,22]. Thus, the consequence of reduced SERCA activity may be impaired Ca^{2+} signaling in the ER and cytoplasm (Figure 1B). Steatosis also impairs the expression of the type II inositol 1,4,5-trisphosphate receptor and

numerous hepatic metabolic genes that, in turn, exacerbate intracellular calcium signaling [9,42]. Recent studies further support the hypothesis that hepatic lipid accumulation is linked to impaired hormonal signaling and Ca^{2+}-dependent metabolic responses (e.g., gluconeogenesis) [31,32].

Mechanisms of Hepatic Steatosis: The Role of Impaired Ca^{2+} Signaling

Alerted Ca^{2+} signaling is one of many factors that contribute to the formation of LDs and steatosis development. Several calcium channels have been identified to be associated with liver metabolic syndrome (Table 1).

Excess Intracellular Lipid Inhibits SOCE via PKC Activation

Through use of the PKC inhibitors GF109203X and calphostin C, and PMA, a PKC activator, PKC has been shown to mediate the lipid-induced inhibition of SOCE [16]. DAG and/or LDs can activate PKC, which in turn leads to PKC-mediated phosphorylation (inhibition) of Orai1, thereby causing inhibition of SOCE in steatotic hepatocytes (shown schematically in Figure 1C).

Inhibition of SOCE Exacerbates Lipid Accumulation

Impaired SOCE has been shown to induce lipid accumulation in steatotic hepatocytes [16], fat storage cells [43], and fat bodies in fruit flies [44,45]. The underlying mechanisms by which impaired SOCE mediated by STIM deficiency regulates adipose tissue lipid content remains unknown [18]. In the liver of obese mice, SERCA2b expression and/or activity is considerably reduced [5,21] which may dysregulate ER Ca^{2+} homeostasis and subsequently trigger an ER stress response [4,34]. Disturbances to Ca^{2+} refilling of the ER may lead to a reduced ER Ca^{2+} content [5] and this low ER Ca^{2+} content may result in increased triglyceride synthesis in the ER membrane lipid bilayer [26]. Reduced ER luminal Ca^{2+} is associated with saturated fatty acid-mediated ER stress [17,46]. Additionally, an increased ER stress response may activate lipogenesis programs in the cytosol, resulting in increased supply of free fatty acids for further DAG and TAG synthesis in the ER [4,5,20,26,47]. A low-Ca^{2+}-initiated ER stress response stimulates the synthesis of DAG and TAG [18,21,43] ultimately leading to enhanced lipid accumulation in the cytoplasmic space. The increased DAGs, on being translocated to the plasma membrane, may activate PKCs, leading to SOCE inhibition. Given a causative

Table 1. Calcium Channels Involved in Metabolic Disorders of the Liver[a]

Calcium channels involved	Effects on liver/hepatocytes	Model	Refs
SOC channels	Reduced intracellular Ca^{2+} may contribute to the development of steatosis	*In vitro* cell culture model; rat hepatocytes Mouse hepatocytes (E. Ali and N. Petrovsky, unpublished), human cells	[7,16,17]
TRPM2 channels	Overexpression of TRPM2 channels is associated with NAFLD	Mouse model	[76]
TRPM7 channels	Inhibition of TRPM7 channels may cause liver fibrosis	Rat hepatic stellate cells	[77]
TRPV4 channels	Upregulation of TRPV4 channels is associated with liver fibrosis	Fibrotic tissues from human liver, HSC-T6 cells	[78]
Ryr1, *Ryr2*, *Cacna1d*, *Cacna1h*, *P2rx1*, and *Itpr1* encoding calcium channel proteins	Dysregulated calcium pathways by mutations and aberrant expression in NASH–HCC	Mouse model	[9]

[a]TRPM, transient receptor potential melastatin; TRPV, transient receptor potential channels of the vanilloid subtype.

relationship between Ca^{2+} influx and lipid accumulation, this suggests that SOCE may be a potential regulator of intracellular lipid synthesis in liver cells. The detailed mechanisms of inhibition of SOCE and associated lipid accumulation are summarized in Figure 2 which shows schematically how DAGs and LDs can be induced under experimental conditions of liver cell

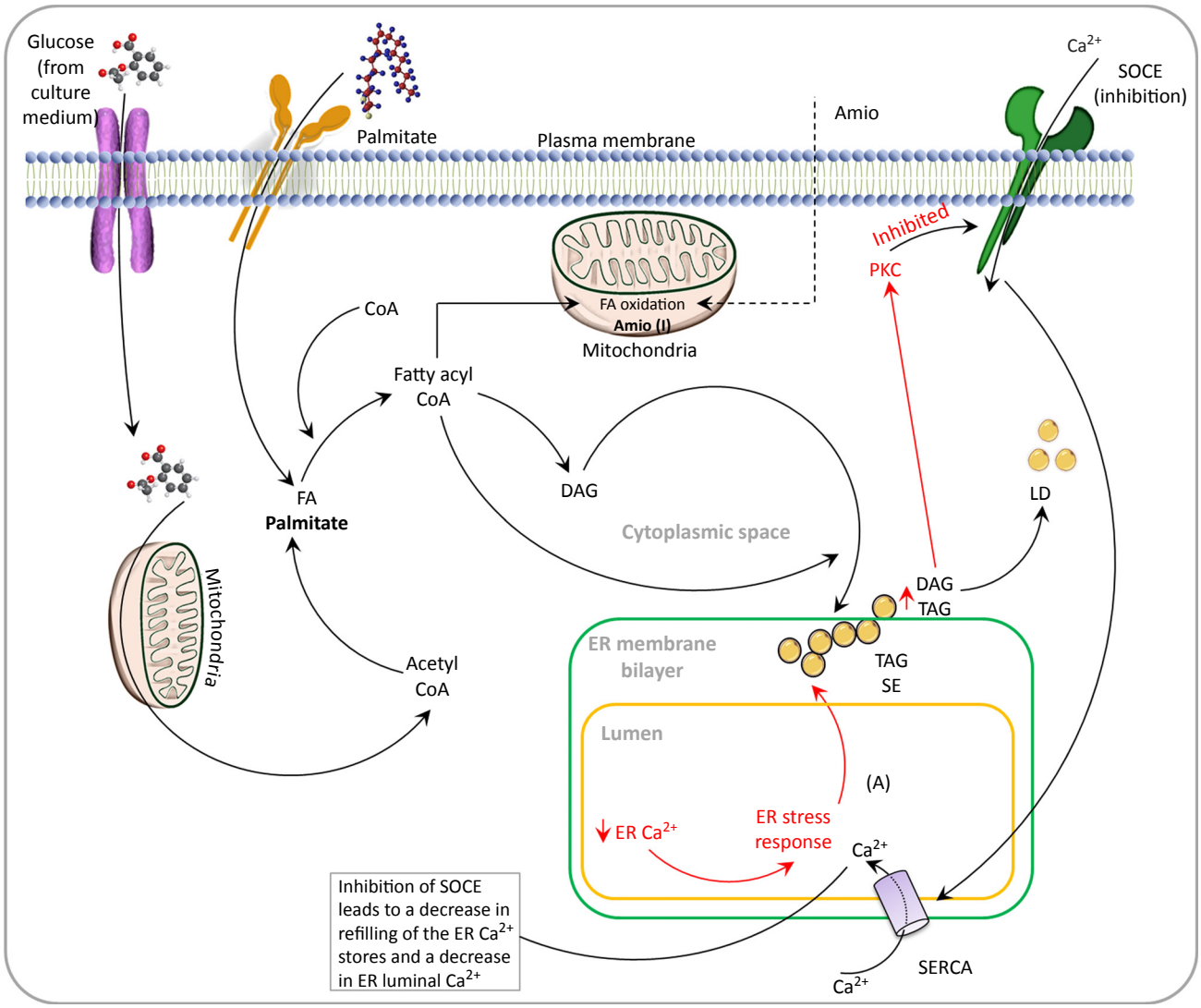

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Figure 2. A Schematic Representation of the Proposed Mechanism By Which Store-Operated Ca^{2+} Entry (SOCE) Inhibition Leads to Enhanced Lipid Accumulation in Hepatocytes in the Presence of Palmitate or Amiodarone. We propose that inhibition of SOCE leads to a decrease in refilling of the endoplasmic reticulum (ER) Ca^{2+} stores and a decrease in ER luminal Ca^{2+}. In the presence of CoA, incorporated palmitate ultimately produces diacylglycerol (DAG). Amiodarone inhibits fatty acid (FA) oxidation in mitochondria [75], which in turn increases FA availability in the cytoplasmic space. These excess FAs can be utilized in the synthesis of DAG and triacylglycerol (TAG) at the ER membrane [26,31]. Low-ER-Ca^{2+}-initiated ER stress response stimulates the synthesis of DAG and TAG [18,21,43] utilizing those excess FAs, which ultimately causes enhanced lipid accumulation in the cytoplasmic space. When palmitate is used as an extracellular source of FAs for lipid synthesis, an excess of palmitate and other FAs (e.g., derived from glucose) is thought to be available in the ER membrane. As stated above, a low-ER-Ca^{2+}-initiated ER stress response stimulates the synthesis of DAG and TAG [16,20] utilizing those excess FAs and palmitate, which ultimately causes enhanced lipid accumulation in the cytoplasmic space. Increased amounts of DAG activate protein kinase C (PKC) [31], which in turn inhibits SOCE through Orai1 phosphorylation [74]. Activation (A) and inhibition (I) are indicated by unbroken lines. SE, sterol ester; LD, lipid droplet.

culture and the mechanism by which SOCE inhibition enhances lipid accumulation in the presence of palmitate or amiodarone.

Pathological Consequences of Inhibition of SOCE

There is increasing evidence that excessive hepatic lipid accumulation and the ER stress response are important factors in the development of insulin resistance and T2D [6,28,31]. Alterations in hepatocyte intracellular Ca^{2+} signaling caused by lipid accumulation or abnormal lipid metabolism may be involved in the transition from simple steatosis to hepatic insulin resistance [22]. Optimal intracellular hepatocyte Ca^{2+} levels are important in the synthesis and post-translational modification of key intracellular signaling proteins involved in insulin pathways. Therefore, intracellular Ca^{2+} homeostasis is crucial for the proper function of insulin. Decreased SOCE into steatotic hepatocytes may alter Ca^{2+}-dependent insulin signaling, leading to hepatic insulin resistance. Figure 3 shows how reduced SOCE in steatotic hepatocytes may lead to hepatic insulin resistance.

Current Treatment Options for Liver Steatosis and Insulin Resistance and the Need for More Specific Therapeutic Strategies

While there is no drug currently approved for the specific treatment of NASH, there are a variety of drugs on the market (reviewed in [48–50]) that are used to treat insulin resistance and T2D and may indirectly reduce steatosis. However, there is an ongoing need for new therapeutic strategies able to directly reverse liver steatosis [50,51].

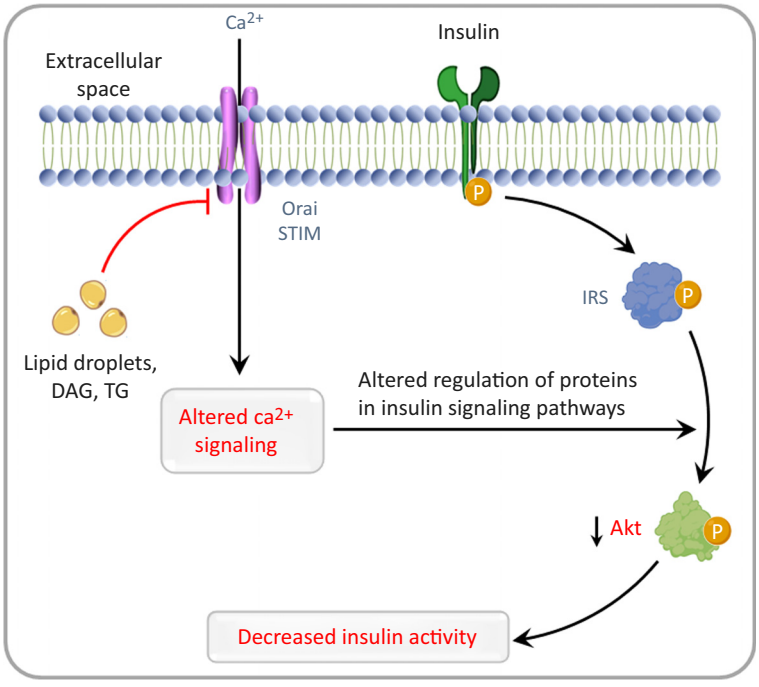

Figure 3. Decreased Ca^{2+} Entry to Steatotic Hepatocytes through Store-Operated Ca^{2+} (SOC) Channels: A Possible Step in the Development of Insulin Resistance. Accumulation of diacylglycerol and lipid droplets in the liver leads to the activation of protein kinase Cε (PKCε), which subsequently inhibits insulin receptor kinase [27,31]. In addition, altered intracellular Ca^{2+} signaling initiated by the inhibition of SOC entry (SOCE) in steatotic liver cells may alter the regulation of key proteins involved in insulin signaling pathways and ultimately decrease insulin activity.

While the insulin receptor, the glucagon receptor, glucose transporters, Akt phosphorylation, PPARγ, and the glucagon-like peptide-1 (GLP-1) receptor are known molecular targets for the treatment of insulin resistance and T2D [51], more recently AMPK, the SERCA protein, cAMP, PKC, and **SOC channels** have been identified as potential targets for the treatment of liver steatosis and insulin resistance [36,52,53]. Existing antidiabetic drugs may interact with intracellular Ca^{2+} signaling pathways, with a recent study suggesting that rosiglitazone, a PPARγ agonist, modulates Ca^{2+} entry through TRP Ca^{2+} channels [54] and induces changes in intracellular Ca^{2+} in smooth muscle cells [55]. GLP-1 analogs have been shown to regulate β-cell insulin secretion through a Ca^{2+}-dependent mechanism [56]. There is also some evidence that metformin can modulate mitochondrial Ca^{2+} transport and intracellular Ca^{2+} signaling [57] and inhibit hormone-induced intracellular Ca^{2+} oscillations [58].

Ca^{2+} signaling, being a universal intracellular messenger, is critical for normal physiology with the potential for its disruption to lead to pathological conditions. Hence, drugs that help to maintain optimal Ca^{2+} homeostasis may be highly beneficial. As many Ca^{2+} channels/transporters play roles in normal cellular functions, a challenge in the development of drugs for NASH is to ensure they are specific to only the relevant Ca^{2+} pathway [19,59].

An important aspect of Ca^{2+} signaling that makes Ca^{2+} channels and pumps attractive as therapeutic targets is the unique spatial and temporal regulation of Ca^{2+} signaling. The Ca^{2+} signal in hepatocytes is usually highly regulated both spatially (in particular regions of the cell) and temporally (by the frequency of Ca^{2+} oscillations) [19,59]. By contrast, in steatotic hepatocytes there is a shift to global elevation of intracellular calcium, making steatotic hepatocytes more susceptible than normal cells to Ca^{2+} channel modulation [19,60]. Another approach could be to directly target specific isoforms of Ca^{2+} channels or pumps associated with NASH [9,59,60]. For example, the expression of the SERCA2b isoform is particularly altered in NASH [21] and may thereby serve as a specific drug target localized to steatotic hepatocytes.

While Ca^{2+} signaling controls many hormonal functions, little work has been done to exploit Ca^{2+} channels as targets for new antiobesity or antidiabetic drugs. Therapeutic agents that directly target inhibited SOC channels and correct the associated ER Ca^{2+} imbalance might offer novel means to treat hepatic steatosis and related metabolic disorders.

Reversal of Impaired SOCE Improves Hepatic Steatosis and Insulin Resistance

Weight loss is the single most effective intervention to reverse hepatic steatosis and hepatic insulin resistance in humans. With a hypocaloric diet and modest weight loss of less than 10% of total body weight, hepatic steatosis improves in obese T2D patients alongside improvements in insulin sensitivity [61]. Since lipid accumulation is associated with impaired SOCE in steatotic hepatocytes [16], agents that act on SOCE to counteract lipid accumulation might improve steatosis. Exendin-4 was shown to prevent the accumulation of lipid in liver cells incubated with palmitate and prevented the increased lipid accumulation caused by pharmacological inhibition of the Ca^{2+} entry channel [17]. By activating SOCE in steatotic hepatocytes, exendin-4 was shown to reduce the total amount of lipid accumulated under conditions where the liver cells are subject to an oversupply of fatty acids [17]. Exendin-4 similarly reversed liver steatosis in *ob/ob* diabetic mice [2]. Pyruvic acid was shown to increase SOCE by reducing the inactivation of Ca^{2+} channels in RBL-1 cells [62]. Oxidation by H_2O_2 or buthionine sulfoximine caused STIM1 to form puncta and open Orai1 channels to activate SOCE [63].

At present, there are no known and reliable small-molecule activators of SOCE. 2-Aminoethyldiphenyl borinate (2-APB) in the 1–20-μM range can activate SOC channels but its

pharmacology is complex and varies by cell type [64]. Future studies are needed to identify '**allosteric**' activators that could potentially bind to the Orai1 polypeptide and activate the channel pore, or that bind with STIM1 or the ER membrane to enhance the oligomerization of STIM1 and its interaction with Orai1. Additionally, selective inhibition of PKC isoforms that inhibit phosphorylation of Orai or the use of taurodeoxycholic acid, which has been shown to activate SOCE in hepatocytes [8], may help to treat NASH. Thrombin and cerulein, activators of SOCE in human lung microvascular endothelial cells [65] and in pancreatic acinar cells [66], respectively, might also be useful if tested in NASH. Similarly, methacholine or platelet-derived growth factor, which activate SOCE in neuronal precursor cells, might be useful to test for treatment of NASH [67]. In a recent study, CDN1163, an allosteric activator of SERCA2b, significantly reduced the hepatic expression of genes involved in gluconeogenesis and lipogenesis and decreased the ER stress response and hepatocyte apoptosis [4,5,52]. Normalizing ER Ca^{2+} flux in *ob/ob* mice by use of the SERCA activator CDN1163 improved hepatic steatosis and corrected metabolic

Table 2. Evolving Compounds and Reagents Regulating Ca^{2+} Signaling, Specifically the SOC Channel and Its Associated Components, in Liver Steatosis or Steatotic Hepatocytes

Agent name	Category by action	Effect on NAFLD or SOC in steatotic liver	Mechanism	Refs
Maresin 1	Activator of SERCA2b	Reduces hepatic steatosis	Increases hepatic Serca2b mRNA expression	[70]
Hepatic stimulator substance (HSS)	Liver growth-inducing peptide	Efficient protection of hepatocytes from cell death as a result of ER stress	Removal of ROS to restore the activity of SERCA	[79]
Matrine	Tetracyclo-quinolizidine alkaloid	Attenuates ER stress	Regulation of SERCA pathway	[80]
Exendin-4	GLP-1 analog	Reduces hepatic lipid content	Activation of cAMP pathway; reduced PKC activity and decreased PKC-mediated phosphorylation of Orai1 resulting in increased SOCE	[8,17]
Calphostin C	PKC inhibitor; perylenequinone metabolite of *Cladosporium cladosporioides*	Increases SOCE in steatotic liver cells	Inhibition of PKC	[16]
GF109203X	PKC inhibitor	Increases SOCE in steatotic liver cells	Inhibition of PKC	[16]
CDN1163	A novel allosteric activator of SERCA2b	Attenuates ER stress response	Possibly through SERCA2-mediated activation of AMP-activated protein kinase pathway	[52]
GLP-1	31-amino-acid peptide hormone; it is an incretin	Reduces hepatic lipid content	Increases production of cAMP; possibly reduces PKC activity and decreased PKC-mediated phosphorylation of Orai1 resulting in increased SOCE	[17]
Dibutyryl cAMP	Cell-permeable cAMP analog	Decreases hepatic lipid content	Activates cAMP-dependent protein kinases; possibly reduces PKC activity and decreased PKC-mediated phosphorylation of Orai1 resulting in increased SOCE	[17]
siRNA against PKCδ	Inhibition of PKCδ	Diminishes lipid-induced ER stress	Stimulates SERCA activity	[72]
Melatonin	Indoleamine	Increases intracellular calcium in the liver of diabetic rats	Regulation of cellular Ca^{2+} homeostasis	[81]
Curcumin	A diarylheptanoid, belongs to curcuminoids group	Decreases hepatic lipid contents	Activates CISD2, and subsequently it enhances SERCA2b activity	[4]
Azoramide	A modulator of unfolded protein response	Induces weight loss	Resolution of ER stress	[82]
Berberine	A compound from protoberberine group of benzylisoquinoline alkaloids	Alleviates hepatic lipid accumulation	Increases ATP-binding cassette transporter A1 through PKCδ pathway	[83]

syndrome features associated with ER stress. These findings are consistent with a previous study where SERCA2b gene transfer [21] or reduction of ER stress by chemical or chaperones in *ob/ob* mice [33,68] reduced lipogenesis and reversed the accumulation of liver fat and insulin resistance. Recently, jaceosidin, a natural compound, was shown to ameliorate ER stress and insulin resistance via upregulation of SERCA2b [69]. Maresin 1, derived from docosahexaenoic acid biosynthesized by macrophages, was also shown to attenuate NAFLD by suppression of ER stress via upregulation of SERCA2b in obese mice [70]. Compounds that regulate Ca^{2+} signaling, specifically the SOC channel and its associated components, and might be useful for treatment of liver steatosis are summarized in Table 2.

Ruboxistaurin, a PKCβ inhibitor, was shown to ameliorate diabetic nephropathy (DN) and showed promising efficacy in several clinical trials of DN and diabetic retinopathy [71]. PKCε **antisense oligonucleotides** therapy improved hepatic insulin signaling and preserved insulin receptor kinase activity in rat models [36]. siRNA-mediated knockdown of PKCδ stimulated SERCA activity in a liver steatosis model and diminished lipid-induced ER stress [72]. PKCε was shown to be activated in the livers of obese diabetic patients, reinforcing its role as a prime NASH therapeutic candidate [73].

Taken together, these data support a major role of impaired Ca^{2+} signaling, together with diacylglycerol activation of PKC, in the mediation of liver ER stress and insulin resistance. They indicate the possibility of treating hepatic steatosis and insulin resistance by reversing this Ca^{2+} imbalance.

Concluding Remarks and Future Perspectives

The development of hepatic steatosis is a multifactorial process that eventually leads to ER stress, insulin resistance, hepatocyte apoptosis, and inflammation, all of which play important roles in the development of NASH, hepatic insulin resistance, and, potentially, T2D. Recent understanding of the role of impaired Ca^{2+} signaling in ER stress-induced hepatic steatosis suggests that altered Ca^{2+} might be a key target for therapeutic approaches to NASH and hepatic insulin resistance. Correction of impaired Ca^{2+} signaling in *ob/ob* mice or steatotic hepatocytes significantly improved hepatic steatosis suggesting that altered calcium signaling might be a promising target for the management of NASH and insulin resistance, although this still needs to be validated in human subjects (see Outstanding Questions). In particular, such therapies might seek to reactivate inhibited SOCE, inhibit PKC, and/or stimulate SERCA activity.

Outstanding Questions

Are there any antidiabetic drugs other than exendin-4 that can reverse impaired SOCE?

Are direct PKC inhibitors, SOCE inducers, or SERCA activators able to reduce hepatic steatosis and NASH in human patients?

Is thrombin, an activator of SOCE in human lung microvascular endothelial cells, able to activate SOCE in liver cells?

Exendin-4 exerts its lipid-lowering effect by the formation of cAMP, the activation of PKA, and lipolysis. Is there any additional mechanism by which exendin-4 decreases lipid accumulation?

Would the activation of SOCE in steatotic hepatocytes, in which SERCA2b is inhibited, lead to an increase in ER Ca^{2+} level?

What are the levels of Ca^{2+} in the cytoplasmic space, the lumen of the ER, and the mitochondrial matrix in steatotic compared with normal hepatocytes?

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