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Review

Deranged hepatocyte intracellular Ca^{2+} homeostasis and the progression of non-alcoholic fatty liver disease to hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths in men, and the sixth in women. Non-alcoholic fatty liver disease (NAFLD) is now one of the major risk factors for HCC. NAFLD, which involves the accumulation of excess lipid in cytoplasmic lipid droplets in hepatocytes, can progress to non-alcoholic steatosis, fibrosis, and HCC. Changes in intracellular Ca^{2+} constitute important signaling pathways for the regulation of lipid and carbohydrate metabolism in normal hepatocytes. Recent studies of steatotic hepatocytes have identified lipid-induced changes in intracellular Ca^{2+}, and have provided evidence that altered Ca^{2+} signaling exacerbates lipid accumulation and may promote HCC. The aims of this review are to summarise current knowledge of the lipid-induced changes in hepatocyte Ca^{2+} homeostasis, to comment on the mechanisms involved, and discuss the pathways leading from altered Ca^{2+} homeostasis to enhanced lipid accumulation and the potential promotion of HCC. In steatotic hepatocytes, lipid inhibits store-operated Ca^{2+} entry and SERCA2b, and activates Ca^{2+} efflux from the endoplasmic reticulum (ER) and its transfer to mitochondria. These changes are associated with changes in Ca^{2+} concentrations in the ER (decreased), cytoplasmic space (increased) and mitochondria (likely increased). They lead to: inhibition of lipolysis, lipid autophagy, lipid oxidation, and lipid secretion; activation of lipogenesis; increased lipid; ER stress, generation of reactive oxygen species (ROS), activation of Ca^{2+}/calmodulin-dependent kinases and activation of transcription factor Nrf2. These all can potentially mediate the transition of NAFLD to HCC. It is concluded that lipid-induced changes in hepatocyte Ca^{2+} homeostasis are important in the initiation and progression of HCC. Further research is desirable to better understand the cause and effect relationships, the time courses and mechanisms involved, and the potential of Ca^{2+} transporters, channels, and binding proteins as targets for pharmacological intervention.

1. Introduction

Hepatocellular carcinoma (HCC) involves the accumulation of mutations in hepatocytes, the principle cell type present in the liver. HCC is an important health issue in both Western and Asian countries [1–3]. Nonalcoholic fatty liver disease (NAFLD) is a major risk factor for the development of HCC [2,3]. Changes in the concentration of Ca^{2+} in the cytoplasmic space, endoplasmic reticulum (ER) and mitochondria are essential for the normal regulation of carbohydrate, lipid and protein metabolism, as well as many other pathways such as the excretion of bile acids [4–6]. Studies conducted over the past 10 years have pointed to significant lipid-induced alterations in Ca^{2+} signaling in steatotic hepatocytes (reviewed in [7–11]). This, in turn, leads to further lipid accumulation and to the creation of an environment which is thought to promote mutagenesis and the progression of HCC [7,11–16] (Fig. 1). The aim of this review is to summarise the changes in intracellular Ca^{2+}

Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B; HCV, hepatitis C; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; $[Ca^{2+}]_{cyt}$, concentration of free Ca^{2+} in the cytoplasmic space; $[Ca^{2+}]_{ER}$, concentration of free Ca^{2+} in the lumen of the ER; $[Ca^{2+}]_{MT}$, concentration of free Ca^{2+} in the mitochondrial matrix; PKC, protein kinase C; PKA, protein kinase A; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CamKK2, Ca^{2+}/calmodulin-dependent protein kinase kinase 2; CaMKIV, Ca^{2+}/calmodulin-dependent protein kinase IV; ER, endoplasmic reticulum; MAM, mitochondrial associated membrane; SERCA, sarco/endoplasmic reticulum ($Ca^{2+}+Mg^{2+}$)ATP-ase; SOCE, store-operated Ca^{2+} entry; CRAC channel, Ca^{2+} release-activated Ca^{2+} channel; I_{CRAC}, current through CRAC channels; VOCC, voltage-operated Ca^{2+} channels; STIM, stromal interaction molecule; TRP, transient receptor potential; $InsP_3$, inositol 14,5-trisphosphate; $InsP_3R$, $InsP_3$ receptor; ROS, reactive oxygen species; Nrf2, nuclear factor erythroid 2-related factor 2; DEN, diethylnitrosamine

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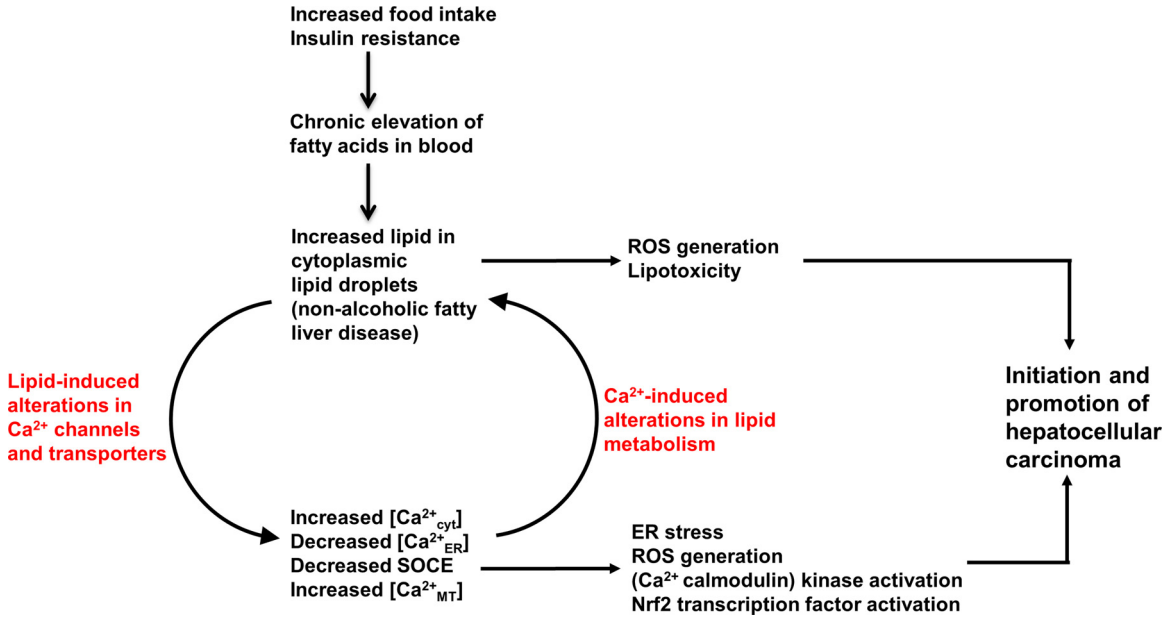

Fig. 1. Schematic overview of the effects of lipid accumulation in steatotic hepatocytes on intracellular Ca^{2+} homeostasis, the proposed positive feed-back cycle whereby altered Ca^{2+} homeostasis in turn exacerbates further lipid accumulation, and the hypothesised pathways (mechanisms) by which altered intracellular Ca^{2+} homeostasis may lead to the development of HCC. ER, endoplasmic reticulum; SOCE, store-operated Ca^{2+} entry; ROS, reactive oxygen species.

signaling caused by lipid accumulation in hepatocytes, to describe the proposed links between altered Ca^{2+} signaling, lipids, and HCC, and to discuss the evidence that lipid-induced changes in intracellular Ca^{2+} signaling promote the development of HCC.

Mutations in many Ca^{2+} transporters, channels, and Ca^{2+} binding proteins have been identified in DNA derived from liver tumors in patients with HCC, and in animal models of HCC [16–18]. Moreover, the expression of a number of Ca^{2+} signaling and Ca^{2+} binding proteins is altered in HCC tissue [16,19–21]. While some of these mutations or changes in protein expression may involve DNA from non-hepatocyte cell types present in liver, the observations suggest that, in the transformed hepatocytes which constitute liver tumors, Ca^{2+} signaling is altered [16]. While these changes in Ca^{2+} signaling are undoubtedly important in the progression and migration of established HCC, the focus of this review is on lipids and the liver environment which is proposed to set up the initiation and promotion of HCC.

2. Hepatocellular carcinoma

The term “primary liver cancer” encompasses HCC, intrahepatic cholangiocarcinoma, fibrolamellar hepatocellular cancer and pediatric hepatoblastoma, all of which originate in the liver [22]. HCC is the second leading cause of cancer-related deaths in men, and the sixth in women, world-wide [1–3]. HCC is often not noticed in the early stages, and is difficult to treat in later stages [1–3]. Major risk factors which pre-dispose to the development of HCC are hepatitis B (HBV), hepatitis C (HCV), environmental toxins (especially aflatoxins) NAFLD, obesity, and type 2 diabetes [2,3]. With the development of greatly improved drugs for the treatment of HBV and HCV, NAFLD is now one of the most important risk factors for HCC [2,23]. Moreover, during the last 10 years or so there has been a substantial increase in the prevalence of NAFLD [2,23].

In the intermediate and advanced stages, the livers of HCC patients exhibit one or more solid tumor nodules and a reduction in functional liver tissue [22,24]. The overall sequence of events in the development of HCC includes induction of hepatocyte injury (eg steatohepatits initiated by HBV, HCV or NAFLD), fibrosis, cirrhosis (but not always), the formation of one or more small tumor nodules, followed by an increase in nodule size and number [22,25]. Advanced stage HCC can be detected by physical symptoms, imaging, and measurement of serum alpha-fetoprotein. In the early stages, HCC can only be detected by screening tests, which often include ultrasound, cross sectional imaging, and measurement of serum alpha-fetoprotein [2,22]. There are several systems for staging HCC, including the Hong Kong liver cancer staging system which includes measurement of the number and size of nodules, and the extent of intrahepatic venous invasion [26].

Genetic mutations in HCC are numerous, depend on the nature of the initiating condition (eg virus, aflatoxin, NASH), and show no clear progression of occurrence of a few key mutations such as found in colon cancer [2,27]. Mutations detected with high frequency include those in genes encoding proteins of the Wnt/beta-catenin pathway, p53 cell cycle pathway, telomere maintenance, and chromatin regulation. As mentioned above there are also numerus mutations in Ca^{2+} signaling pathways [16,22,25]. In addition, changes in expression of many genes have been observed, including those in Ca^{2+} signaling pathways [16,19–21]. Epigenetic modification has been shown to be involved [28,29].

The origin of HCC cells is complex, closely linked to the regeneration, formation and differentiation of the various cell types in liver, and not yet fully understood. It is presently thought that adult hepatocytes are the principal cell of origin of HCC cells, although there is evidence that hepatic progenitor cells are also precursors of HCC cells [22,25]. Under appropriate pathological conditions, such as HBV or HCV viral infection, potential mutagens such as aflatoxin, and/or cell injury as in non alcoholic steatohepatitis (NASH), adult hepatocytes (and hepatic progenitor cells) acquire a series of genetic mutations, de-differentiate, and are transformed into HCC cells. The mutations allow the transformed cells to grow faster than resident hepatocytes which leads to the expansion of colonies of mutated cells [22,25]. As well as the accumulation of mutations, progression of HCC to later stages is greatly enhanced by the metabolic environment of the liver, which includes lipotoxicity, oxidative stress, endoplasmic reticulum (ER) stress, and inflammation. These factors act essentially as tumor promoters [2,27].

The front-line treatment for HCC is surgical liver resection or liver transplantation [24]. Where surgical intervention is not possible, other available treatments include liver directed radiofrequency tumor ablation, delivery of chemotherapeutic agents to the exact location of a tumor nodule by transcatheter arterial chemoembolization (TACE), and

systemic treatment with statins, metformin, protein tyrosine kinase inhibitors, or PD-1 (programmed death-ligand 1) receptor blockers [24]. Unfortunately, these non-surgical treatments do not provide a cure for HCC, and even surgical treatment may only be temporary due to the possibility of HCC recurrence. Non-surgical treatments normally can only increase patient survival for a short period of time and improve quality of life during that period.

As just implied, HCC is a complex cancer, is not readily apparent in its early stages, and is difficult to treat. There is a clinical need for gaining a better understanding of the underlying molecular pathology of HCC, to improve the ability to identify at an early stage subjects predisposed to HCC, and to develop new strategies for prevention [2,30]. This is especially the case for NAFLD subjects pre-disposed to HCC. It is predicted that that in 2025 NAFLD will be the leading risk factor for HCC [2].

3. Non-alcoholic fatty liver disease as a risk factor for hepatocellular carcinoma

The term non alcoholic fatty liver disease refers to a spectrum of liver disorders which range from the accumulation of excess lipid in hepatocytes (simple steatosis) to NASH in which lipid accumulation over time leads to hepatocyte injury, inflammation, and peri-cellular fibrosis [16,31–33]. The progression from simple steatosis to NASH is principally mediated by toxic free fatty acids (lipotoxicity) and an increase in reactive oxygen species (ROS) (oxidative stress) [16,31–33]. About 10 to 30% of NAFLD subjects have NASH [16,31–33].

Lipid metabolism in hepatocytes involves several pathways [34] (Fig. 2A). Fatty acids are synthesized *de novo* from acetyl-CoA through the lipogenic pathway in the ER, esterified to diacyl glycerol (DAG) and triacyl glycerol (TAG), and, together with cholesterol, are excreted to the systemic circulation in very low density lipoproteins. Hepatocytes take up lipids and cholesterol from chylomicrons in the blood following a meal and can take up free fatty acids from the systemic circulation when these are released from peripheral tissues, chiefly adipose tissue. The oxidation of lipids by the beta-oxidation pathway in mitochondria is an important source of energy (ATP synthesis) for hepatocytes. Another component of the hepatocyte lipid metabolism system is the storage of DAG and TAG in cytoplasmic lipid droplets [35,36]. These are formed on the cytoplasmic side of the ER and bud off into the cytoplasmic space (Fig. 2 A and B). Fatty acids can be released from TAG and DAG originating in cytoplasmic lipid droplets by hydrolysis by cytoplasmic lipases [37]. In addition, cytoplasmic lipid droplets can be removed from the cytoplasmic space by autophagy, which delivers the lipid droplet and its contents to a lysosome [38].

Under normal conditions hepatocyte fatty acid uptake, *de novo* lipid synthesis, and lipid oxidation, excretion, and storage are balanced (Fig. 2C). However, in obese subjects, who are also often insulin resistant, the flow of fatty acids from the intestine, adipose tissue, and other peripheral tissues to hepatocytes is greater than that needed for beta oxidation and ATP generation. Under these conditions, the excess fatty acids accumulate as TAG in cytoplasmic lipid droplets [31,33,35,37] (Fig. 2C). An example of cytoplasmic lipid droplets in steatotic hepatocytes isolated for a genetically obese Zucker rat is shown in Fig. 2D (fat) and 2E (lean, no lipid droplets). Storage of pathological amounts of TAG in lipid droplets provides a reservoir of potentially toxic saturated fatty acids, as well as DAG which can activate protein kinase C (PKC).

If not controlled, NAFLD can progress to NASH, fibrosis and cirrhosis, and then, under appropriate conditions, to HCC [23,39–41]. Often HCC can develop without cirrhosis, suggesting that the mechanisms involved in development of HCC in NAFLD subjects may differ somewhat from those involved in the development of HCC from HBV and HCV (Fujiwara et al 2018). Factors present in NAFLD livers which are thought to mediate the development of HCC include oxidative stress, lipotoxicity, ER stress, the generation of pro-inflammatory cytokines and adipokines, and changes in intestinal microbiota [2,29,40,42,43]. These factors provide an environment in the liver suitable for the promotion and progression of HCC. Lipotoxicity can be defined as chronically elevated concentrations of certain lipids, including un-esterified cholesterol, saturated free fatty acids, diacylglycerols, lysophosphatidyl-choline, sphingolipids, ceramide, leukotrienes and/or prostaglandins. Elevated concentrations of these lipids cause damage to membranes and organelles [42,44,45].

4. Changes in endoplasmic reticulum and mitochondrial calcium homeostasis in steatotic hepatocytes

Results obtained in a number of laboratories have shown that lipid accumulation in steatotic hepatocytes causes substantial changes in intracellular Ca^{2+} signaling (summarized, with references, in Table 1). The Ca^{2+} channels and transporters which have so far been identified as being altered in steatotic hepatocytes, and which have been studied in some detail, are the sarco/endoplasmic reticulum ($Ca^{2+} + Mg^{2+}$)ATP-ase2b (SERCA2b), the type 1 inositol trisphosphate receptor ($InsP_3R1$), and store-operated Ca^{2+} entry (SOCE) channels. The main lipid-induced changes in intracellular Ca^{2+} concentrations are a decrease in the free Ca^{2+} concentration in the ER ($[Ca^{2+}]_{ER}$), and increases in the free Ca^{2+} concentrations in the cytoplasmic space ($[Ca^{2+}]_{cyt}$) and mitochondrial matrix ($[Ca^{2+}]_{MT}$). Early studies provided evidence that the observed lipid-induced decrease in SERCA2b activity is due to inhibition of the catalytic activity of SERCA2b by fatty acids and phospholipids present in the membrane, and to decreased expression of SERCA2b protein [46–48]. Analysis of the lipid composition of ER membranes isolated from the livers of obese mice provided evidence that the lipid-induced inhibition of the catalytic activity of SERCA2b is due to an enrichment of monosaturated fatty acids and an increase in the phosphatidyl choline:phosphatidylethanolamine ratio, especially the elevated levels of phosphatidyl choline [46]. The actions of phosphatidyl choline may be due to a direct effect of phosphatidyl choline on the SERCA2b protein, and/or to altered fluidity of the ER membrane, as suggested by a recent study of the role of mitochondria-associated thioesterase superfamily member 2 (Them 2) in affecting fluidity of the ER membrane [8].

Additional insights into the lipid induced inhibition of SERCA2b in hepatocytes have come from studies of Cisd2 haploinsufficiency [9]. Cisd2 is an integral membrane protein found in the ER membrane and in the mitochondrial outer membrane [9]. Using genetically modified mice, Shen et al 2017 found that the livers of mice with Cisd2 haploinsufficiency exhibit decreased $[Ca^{2+}]_{ER}$, increased $[Ca^{2+}]_{cyt}$, and increased $[Ca^{2+}]_{MT}$ (Fig. 3A–E). These changes in Ca^{2+} were associated with altered morphology of the ER and mitochondria (Fig. 3F). The results indicate that the Cisd2 protein is necessary for normal SERCA2b activity. The authors proposed that Cisd2 normally binds to, and activates, SERCA2b, possibly by affecting the oxidative modification of SERCA2b.

A decrease in $[Ca^{2+}]_{ER}$ in steatotic hepatocytes has also been detected *in vivo* using an indirect approach involving use of a luminescent reporter protein (Gluc-SERCaMP) located in hepatocytes [49]. This reporter construct normally resides in the lumen of the ER. A decrease in $[Ca^{2+}]_{ER}$ initiates secretion of Gluc-SERCaMP from the lumen of the ER and then from the hepatocyte to the blood where it can be detected as an increase in blood plasma luminescence [49]. Using viral-mediated delivery of DNA encoding Gluc-SERCaMP, the protein reporter was expressed in the livers of rats. The approach was validated by administration of thapsigargin to inhibit SERCA2b and induce Ca^{2+} release from the ER (Fig. 4A). Switching the diet of rats from one restricted in calories to a high caloric diet was associated with an increase in blood luminescence, consistent with lipid-induced inhibition of SERCA2b leading to a decrease in $[Ca^{2+}]_{ER}$ (Fig. 4B,C). The release of Gluc-SERCaMP to the blood induced by the high caloric diet was associated with decreased SERCA2b (ATP-ase) activity (but no change in SERCA2b

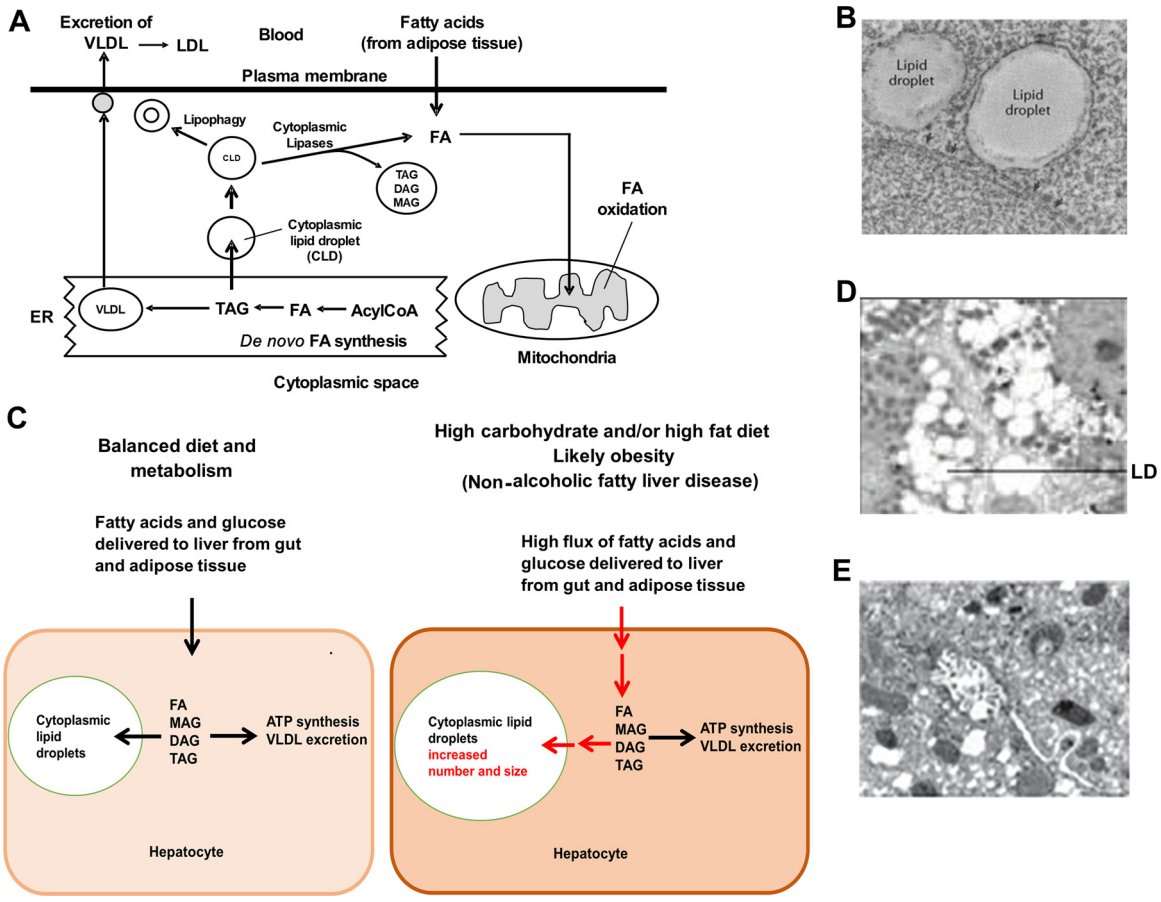

Fig. 2. Pathways of lipid metabolism, and the storage of lipid in cytoplasmic lipid droplets in hepatocytes. A. Schematic representation of the main pathways of lipid metabolism and storage in hepatocytes. B. Emergence of lipid droplets from the endoplasmic reticulum in yeast. A 2D tomogram showing nuclear ER membrane (bilayer) and the membrane of the lipid droplets (monolayer) in which phospholipids are orientated with their polar head groups towards the cytoplasmic space and their acyl chains towards the hydrophobic neutral lipid core of the droplet (From Olzmann and Carvalho [36]). C. Schematic representation of the effect of a chronic elevated supply of fatty acids (principally from diet and adipose tissue) to the liver in a subject with NAFLD. Free fatty acids delivered to hepatocytes in NAFLD exceed the biosynthetic and energy requirements of hepatocytes and are stored as triacyl- and diacyl-glycerol in cytoplasmic lipid droplets. D. and E. Electron micrographs of sections of the livers of genetically obese (D) and lean control (E) Zucker rats, showing the presence of lipid droplets (LD) in the steatotic liver (From Steenks et al. [119]). VLDL, very low density lipoproteins; LDL, low density lipoproteins; FA, free fatty acids; and TAG, DAG and MAG, tri- di- and mono- acyl-glycerols, respectively.

Table 1
Observed changes in Ca^{2+} transporters, channels, and intracellular Ca^{2+} concentrations in steatotic hepatocytes (compared to non-steatotic hepatocytes).

Observation	Comments	References
Inhibition of SERCA2b	Decreased catalytic activity of SERCA2b, measured in microsomes	[47]
	Decreased expression of SERCA2b protein	[46,120] [49]
Inhibition of store-operated Ca^{2+} entry	Proposed PKC-mediated inhibition of Orai1 activity	[7]
	Decreased STIM1 activation	[75] [11]
Increase in $InsP_3R1$ activity and/or expression	Lipid-induced activation of $InsP_3R1$ may involve cyclic AMP- and PKA-mediated phosphorylation and activation of $InsP_3R1$.	[53] [51] [55]
Increase in number of mitochondrial attached membranes (MAMs)	Direct measurements, and inferred from increase in $InsP_3R1$ expression	[51] [55]
Decrease in $[Ca^{2+}]_{ER}$	Results from decreased SERCA2b and SOCE, and increased $InsP_3R1$ and translocon activity	[9,48] [7,8,49]
Increase in $[Ca^{2+}]_{cyt}$	Limited number of direct measurements of lipid-induced increase in $[Ca^{2+}]_{cyt}$	[9]
	Increase in $[Ca^{2+}]_{cyt}$ inferred from changes in SERCA2b and $InsP_3R1$ activity	[13] [111]
Increase in $[Ca^{2+}]_{MT}$	Few direct measurements. Inferred from increased MAMs, outflow of Ca^{2+} from ER, and increased $InsP_3R1$ activity	[9,55] [121]

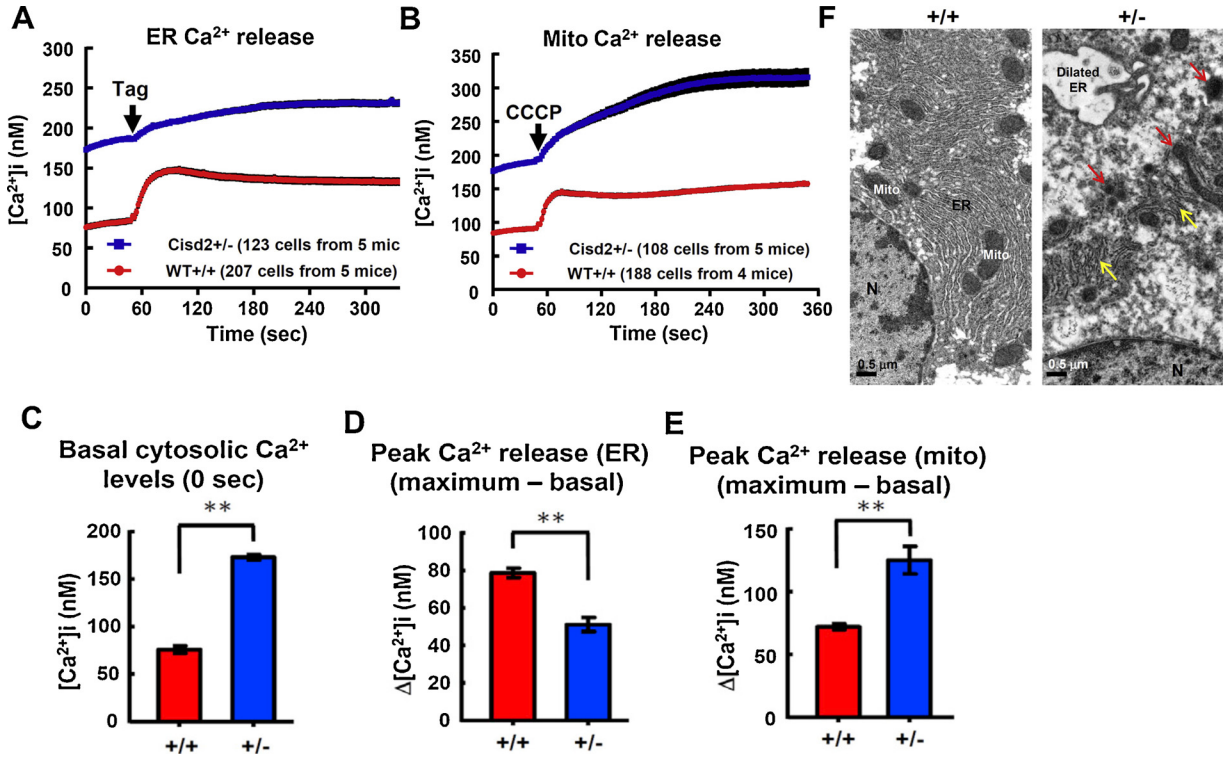

Fig. 3. Effects of SERCA2b inhibition, induced by Cisd2 haploinsufficiency, on the concentrations of Ca^{2+} in the cytoplasmic space (A,B and C), ER (A and D) and mitochondria (B and E), and on the morphology of the ER and mitochondria (F). A-E. The concentrations of Ca^{2+} in the ER and mitochondria were assessed indirectly by measuring the peak of Ca^{2+} release following the addition of thapsigargin (A) or CCCP (B) to hepatocytes isolated from wild type mice (+/+) and mice with Cisd2 haploinsufficiency (+/-). F. Transmission electron micrographs of hepatocytes isolated from wild type mice (+/+) and mice with Cisd2 haploinsufficiency (+/-). Fragmented rough ER is indicated by yellow arrows, and dysmorphic and/or degenerated mitochondria by red arrows (From Shen et al. [9]).

protein expression), measured in isolated microsomes. Release of Gluc-SERCaMP induced by the high caloric diet was somewhat diminished by co-administration of dantrolene, an inhibitor of ryanodine receptors, during the period of high fat diet. As discussed by the authors, interpretation of the results obtained with dantrolene is potentially complex [49]. Moreover, the extent to which hepatocytes express functional ryanodine receptors is not really clear [50].

Several studies have provided evidence which implies that, in steatotic hepatocytes, $InsP_3R1$ receptors are activated (eg by cyclic AMP) and/or expression of the $InsP_3R1$ protein is increased (Table 1)

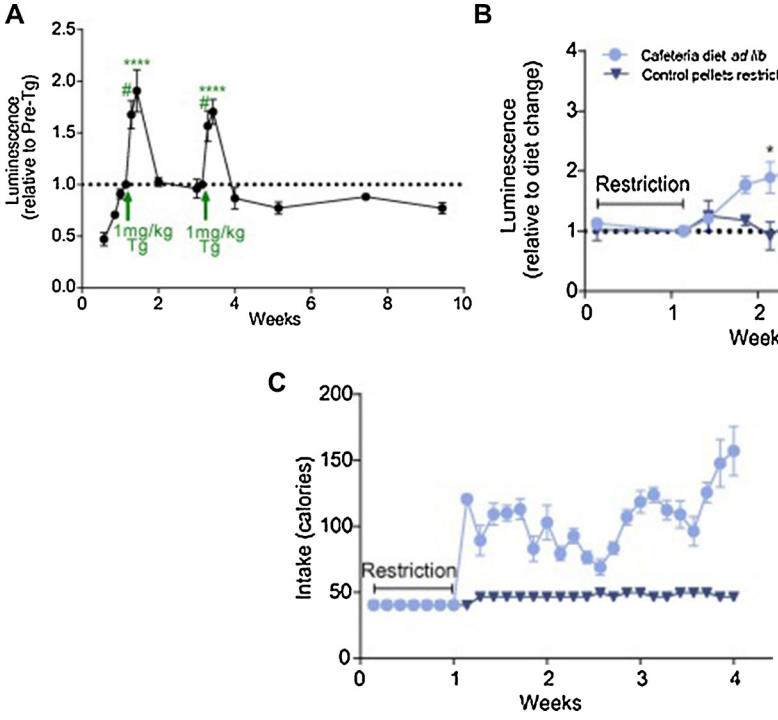

Fig. 4. Lipid-induced decreases in the concentration of Ca^{2+} in the lumen of the ER *in vivo* measured indirectly using the luminescent ER Ca^{2+} reporter Gluc-SERCaMP which can be detected in the blood by luminometry. A. Validation of the methodology using thapsigargin (Tg) (administered by intraperitoneal (i.p.) injection. B. The effect of a cafeteria (high caloric) diet compared with a normal diet on luminescence measured in the blood. C. Calorie intakes for the cafeteria and control diets. The Gluc-SERCaMP construct consists of the final seven amino acids (ASARTDL) of mesencephalic astrocyte-derived neurotrophic factor (MANF), which act as an ER retention sequence, linked to Gaussia luciferase (GLuc) to create a secreted, ER Ca^{2+} modulated protein (SERCaMP). The ASARTDL sequence detects a decrease in $[Ca^{2+}]_{ER}$ which then initiates secretion of Gluc-SERCaMP from the lumen of the ER and from the hepatocyte to the blood. Using viral-mediated delivery of DNA encoding Gluc-SERCaMP, the protein reporter was expressed in the livers of rats. (From Wires et al. [49]).

[51–53]. Hepatocytes principally express $InsP_3R1$ and $InsP_3R2$ (reviewed in [4,5,54]). The latter mediate increases in $[Ca^{2+}]_{cyt}$ involved in the regulation of several hepatocyte functions, including bile secretion [5]. $InsP_3R1$ are involved in hormonal regulation of metabolic pathways, in ER-mitochondria Ca^{2+} transfer across mitochondrial associated membranes (MAMs) and may also be responsible for Ca^{2+} release from that part of the ER involved in the activation of STIM1 and SOCE [4,5,51,54,55].

The evidence that Ca^{2+} efflux from the ER via $InsP_3R1$ may be increased in steatotic hepatocytes compared to non steatotic cells is somewhat indirect. It is principally based on the observations that, in insulin-resistant hepatocytes, increased cyclic AMP and activation of protein kinase A (PKA) phosphorylate and active $InsP_3R1$ [53], and that in obese mice, knockdown of $InsP_3R1$ improves insulin sensitivity [55]. Additional evidence for a role for $InsP_3R1$ in lipid-induced reduction in $[Ca^{2+}]_{ER}$ has come from studies employing genetically modified mice in which expression of $InsP_3R1$ in liver was specifically knocked out [51]. In animals fed a high fat diet, $InsP_3R1$ KO mice exhibited less liver lipid than wild type controls, due, in part, to decreased lipogenesis. These mice exhibited decreased formation of triglyceride and lipid droplets in liver and were resistant to the development of fatty liver. This was attributed to the role $InsP_3R1$ plays in ER-mitochondrial connections [51]. Analysis of liver biopsies from patients with NASH found higher levels of $InsP_3R1$ expression and a higher degree of ER-mitochondrial co-localisation in NASH subjects compared with controls [51]. Taken together, the results of studies of expression and activity of $InsP_3R1$ in steatotic hepatocytes suggest this is likely elevated compared to normal, and contributes to the observed lipid-induced decrease in $[Ca^{2+}]_{ER}$.

Expression of $InsP_3R2$ is also increased in steatotic hepatocytes [56]. However, this increase in $InsP_3R2$ appears to be related to the regulation of liver regeneration rather than to lipid-induced changes in $[Ca^{2+}]_{ER}$.

Under normal conditions, translocons also mediate the efflux of some Ca^{2+} from the ER [57]. Ersoy et al 2018 have shown that, under conditions of high nutrient uptake, thioesterase superfamily member 2 (Them2) and phosphatidyl transfer protein (PCTP), which interacts with Them2, facilitate the incorporation of saturated fatty acids into phospholipids in the ER membrane [8]. The experiments employed mice in which Them2 and PCTP were both knocked out, isolated hepatocytes, and HEK cells. Evidence was provided to indicate that the increased saturated fatty acid content of membrane phospholipids enhances the loss of Ca^{2+}_{ER} to the cytoplasmic space via translocons. Taken together, all the results described above indicate that lipids in steatotic hepatocytes cause a decrease in $[Ca^{2+}]_{ER}$ by both inhibiting Ca^{2+} uptake via SERCA2b, and by enhancing Ca^{2+} efflux via $InsP_3R1$ and translocons.

Lipid-induced changes in the interaction of the ER with mitochondria through MAMs and in the transfer of Ca^{2+} from the ER to mitochondria through $InsP_3R1$ and MAMs also play an important role in lipid induced disruption of intracellular Ca^{2+} homeostasis in steatotic hepatocytes (Table 1). In studying this question, Arruda et al 2014 employed genetically obese mice (ob/ob) and mice fed a high fat diet, electron microscopy of liver sections, and measurement of $[Ca^{2+}]_{MT}$, membrane potential and oxidative phosphorylation in isolated mouse hepatocytes [55]. From their results, the authors concluded that liver steatosis leads to an increase in the number of MAMs and to a pathological increase in mitochondrial Ca^{2+} (overload), leading to decreased oxidative phosphorylation and increased ROS. These conclusions were consistent with the results of other experiments which showed that the knockdown of $InsP_3R1$ in hepatocytes isolated from obese mice improved mitochondrial oxidative phosphorylation [55]. As mentioned above, Feriod et al. found increased expression of $InsP_3R1$ and increased MAMs in liver biopsy samples from NASH patients [51]. In a related study, Rieusset and colleagues have found that disruption of MAMs and movement of Ca^{2+} from the ER to mitochondria are associated with ER stress, dysfunction of mitochondria, accumulation of lipid and insulin resistance [10]. Notwithstanding these interesting results, the effects of steatosis on MAMs, $[Ca^{2+}]_{MT}$, and mitochondrial function appear to be complex and not yet fully understood.

5. Changes in store-operated Ca^{2+} entry in steatotic hepatocytes

In addition to the effects of intracellular lipid accumulation on the ER, SERCA2b, $InsP_3R1$ and on the transfer of Ca^{2+} between the ER and mitochondria, lipid accumulation in steatotic hepatocytes also inhibits SOCE (Table 1). SOCE is one of the main pathways of Ca^{2+} entry to hepatocytes, and plays a central role in maintaining adequate concentrations of $[Ca^{2+}]_{ER}$ under conditions of hormonal Ca^{2+} signaling involving $InsP_3R$-mediated ER Ca^{2+} efflux and Ca^{2+} extrusion from the cell via plasma membrane (Ca^{2+} + Mg^{2+}) ATP-ases (reviewed in [6]). Hepatocytes also express a number of non-selective cation channels, including transient receptor potential (TRP) channels, which are thought to perform specific functions in the regulation of hepatocyte metabolism and physiology (reviewed in [6]).

The electrophysiological properties of SOCE channels in hepatocytes isolated from rat livers are essentially identical to those of CRAC channels expressed in lymphocytes and mast cells (reviewed in [6]). SOCE channels in rat hepatocytes are principally composed of STIM1 and Orai1 proteins, although both STIM1 and STIM2 and all three isoforms of Orai1 are expressed in rat hepatocytes [58–60]. Studies with human hepatocytes suggest that transient receptor potential canonical 6 (TRPC6) channels may also make a contribution to SOCE in these cells [61]).

To our knowledge, there are no reports of studies where the authors have both identified functional voltage-operated Ca^{2+} channels (VOCCs) in isolated hepatocytes and have characterised these using electrophysiological and/or Ca^{2+} imaging techniques. There are several reports of studies where the authors failed to find VOCCs in isolated hepatocytes or liver cells [62–65]. As shown in Fig. 5A, functional VOCCs can be clearly distinguished from SOCE channels (CRAC channels) using Ca^{2+} imaging [66]. The use of VOCC blockers of the dihydropyridine, phenylalkylamine, and benzothiazepine families to detect VOCCs in isolated hepatocytes and in liver cells is complicated since these blockers may also inhibit SOCE at relatively low concentrations [67,68]. Inhibition of SOCE (I_{CRAC}) in isolated rat hepatocytes by the non-selective VOCC blocker mibefradil, and the dihydropyridine efonidipine is shown in Fig. 5B. Several studies have shown that mRNA encoding subunits of VOCCs, or the subunit polypeptides, are expressed in extracts of mouse and human liver [13,69,70]. However, it has been clearly shown that VOCCs are expressed in some other cell types present in liver, including stellate, Kupffer and smooth muscle cells [71–74]. Therefore, the observation that VOCC subunits are expressed in intact liver tissue does not necessarily imply that these channels are expressed in hepatocytes. Expression of VOCC subunits has also been detected in extracts of HepG2 cells, which are derived from a human liver tumour [13,70]. However, HepG2 cells are substantially de-differentiated compared to normal adult hepatocytes, so the observation of expression of VOCC subunits in HepG2 cells does not necessarily imply that these channels are also expressed in normal non-transformed hepatocytes. However, this observation raises the interesting possibility that VOCCs may be expressed in hepatic progenitor cells and/or in hepatic stem cells.

In studies conducted with H4IIE rat liver cells loaded with lipid by treatment with palmitate or amiodarone SOCE was found to be severely inhibited. Similar lipid-induced inhibition of SOCE was observed in hepatocytes isolated from genetically obese Zucker rats and in lean controls [7]. In these studies, SOCE (hepatocyte I_{CRAC}) was measured by patch clamp recording as well as by fluorescence imaging, using Fura-2. Measurement of the amount of Ca^{2+} released from the ER by DBHQ indicated that in these lipid-loaded and steatotic cells the amount of Ca^{2+} in the ER was also greatly reduced. Lipid-induced inhibition of

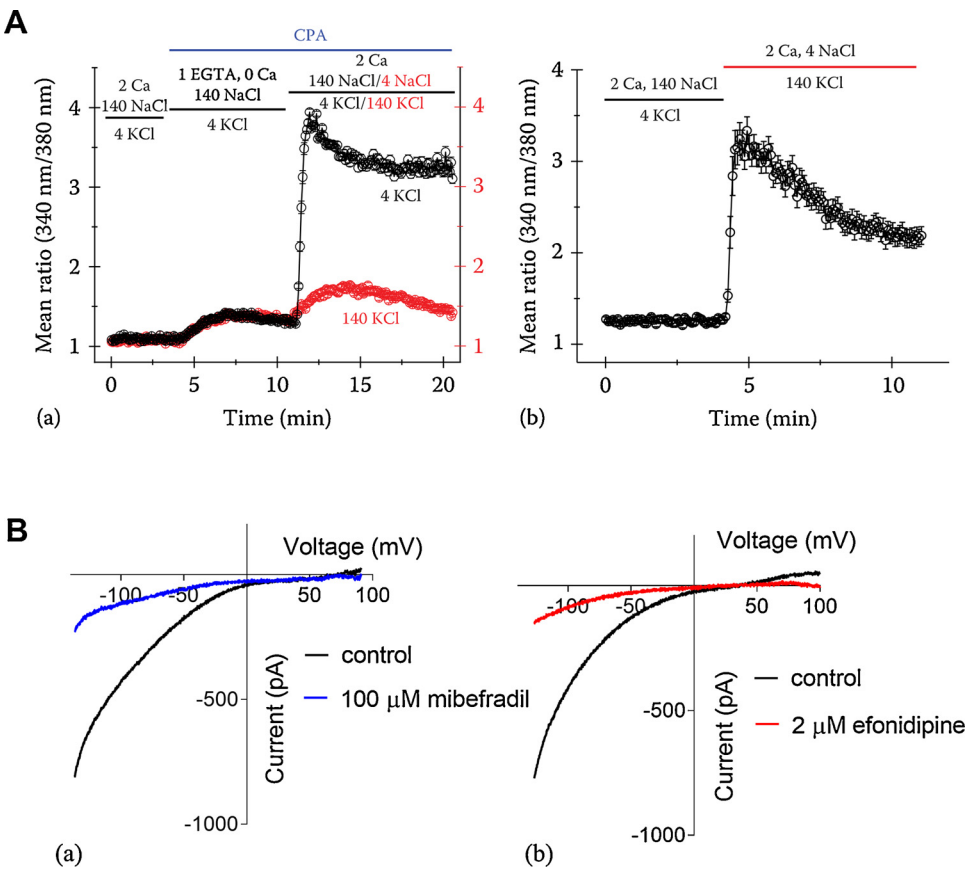

Fig. 5. Differentiation of store-operated Ca^{2+} entry and voltage-operated Ca^{2+} entry. A. Fura-2 measurement of $[Ca^{2+}]_{cyt}$ in human Jurkat T lymphocytes (a) and murine MIN6 β cells (b). In (a) Ca^{2+} entry through endogenous Orai channels was measured using the Ca^{2+} re-addition protocol after ER store depletion with 20 µM cyclopiazonic acid (CPA). External $[K^+]$ was either 4 mM (shown in black) or was elevated to 140 mM (in red) after Ca^{2+} was re-introduced to the bath. In (b) Ca^{2+} entry through endogenous voltage-operated Ca^{2+} channels was evoked by $[K^+]$ elevation in the bathing solution (as in (a)). (From [66]). B. Inhibition, by the non-selective VOCC blocker mibefradil, and by the dihydropyridine VOCC blocker efonidipine, of SOCE (I_{CRAC}) measured in isolated rat hepatocytes using patch clap recording. Currents were recorded in response to voltage ramps between -120 and 120 mV before and after the addition of the blockers to the bath in HEK293 T cells transiently transfected with hOrai1 and hSTIM1 cDNAs. (Rychkov G. and Barritt G. *J.* unpublished results).

SOCE could be reversed by inhibitors of PKC, and also by incubation of the cells with the anti-diabetic drug exendin-4, a GLP-1 (glucagon-like peptide-1) analogue [7,75].

The mechanism by which lipid inhibits hepatocyte SOCE was proposed to involve the activation of one or more isoforms of DAG-sensitive PKC by DAG present in cytoplasmic lipid droplets, phosphorylation of Orai1, and inhibition of Orai1 activity [7,75] (shown schematically in Fig.6A). Previous studies in several laboratories have shown that, of all the DAG-sensitive PKC isoforms expressed in hepatocytes, lipid accumulation is associated with the activation of one or more of PKCε, PKCδ, and PKCβ, depending on the experimental model employed [76–79]. Moreover, in previous studies involving HEK293 cells, it was shown that Orai1 and SOCE can be inhibited by activation of PKCβ, which phosphorylates the N-terminal Ser-27 and Ser-30 residues of Orai1 [80]. Thus lipid-induced inhibition of hepatocyte SOCE may involve PKCβ-mediated phosphorylation of Ser-27 and Ser-30 of Orai1.

The proposed roles of DAG and PKC in the mechanism by which lipid induces inhibition of SOCE (shown schematically in Fig. 6), are likely a simplification of a complex system of interacting pathways. For example, hormonal activation of SOCE involves the activation of phospholipase C and generation of $InsP_3$ which, in turn, releases Ca^{2+} from the ER and provides the signal for initiating the interaction of STIM1 with Orai1 and activation of SOCE [50]. Phospholipase C activation generates DAG in parallel with $InsP_3$ and this DAG could also potentially activate PKC and inhibit Orai1. Hormone-induced increases in DAG may be relatively short-lived whereas in lipid-loaded/steatotic hepatocytes, DAG present in cytoplasmic lipid droplets represents a chronic elevation of DAG and chronic activation of PKC. In these hepatocytes, where $[Ca^{2+}]_{ER}$ is chronically low, SOCE may be active in the absence of hormones, as suggested below. Hormone-induced increases in DAG originate in the plasma membrane, whereas in lipid-loaded cells, DAG likely originates from the esterification of fatty acids delivered from the blood, and/or from DAG in cytoplasmic lipid droplets [78]. Further experiments are needed to resolve these issues, and to clarify the mechanism by which lipid leads to SOCE inhibition.

On the basis that $[Ca^{2+}]_{ER}$ is known to be depleted in steatotic hepatocytes and, as mentioned above, that one of the main functions of SOCE is to maintain adequate $[Ca^{2+}]_{ER}$, Arruda et al. investigated whether lipid accumulation alters the ability of STIM1 to activate SOCE [11]. In studies of the distribution of STIM1 in Lep ob/ob obese mice, they observed an aberrant intracellular distribution of STIM1, and aberrant translocation of STIM1 to the plasma membrane upon depletion of $[Ca^{2+}]_{ER}$. They also observed a high level of O-GlcNAcylation of STIM1, which is known to impair the translocation of STIM1 to the plasma membrane [81]. Arruda and colleagues proposed that lipid-induced O-GlcNAcylation of STIM1 is responsible for the altered intracellular distribution of STIM1 in steatotic hepatocytes, and for the reduced ability of STIM1 to activate Orai1 in these cells.

Two other observations point to additional mechanisms which might be involved in lipid-induced inhibition of SOCE. In experiments employing HEK293 cells, it was shown that chemical-induced depletion of membrane cholesterol enhanced SOCE, suggesting that SOCE may be inhibited when the concentration of cholesterol in the plasma membrane is increased [82]. In a study employing RBL-2H3 mast cells, Holowka et al. showed that polyunsaturated fatty acids inhibit SOCE [83]. The mechanisms are thought to involve the inhibition of Orai1 by cholesterol and the inhibition of the coupling of STIM1 to Orai1 by that polyunsaturated fatty acids.

As summarized in Table 1, lipid accumulation in steatotic hepatocytes is thought to alter intracellular Ca^{2+} homeostasis and Ca^{2+} signaling by inhibiting Ca^{2+} uptake to the ER, inhibiting SOCE (which refills ER Ca^{2+}), enhancing Ca^{2+} efflux from the ER, and enhancing Ca^{2+} uptake by mitochondria. These changes in Ca^{2+} movement are thought to result in, among other things, chronic changes in in ER Ca^{2+} (decrease), $[Ca^{2+}]_{cyt}$ (increase) and $[Ca^{2+}]_{MT}$(increase). Chronic elevation of $[Ca^{2+}]_{cyt}$ when maximum SOCE is substantially reduced may

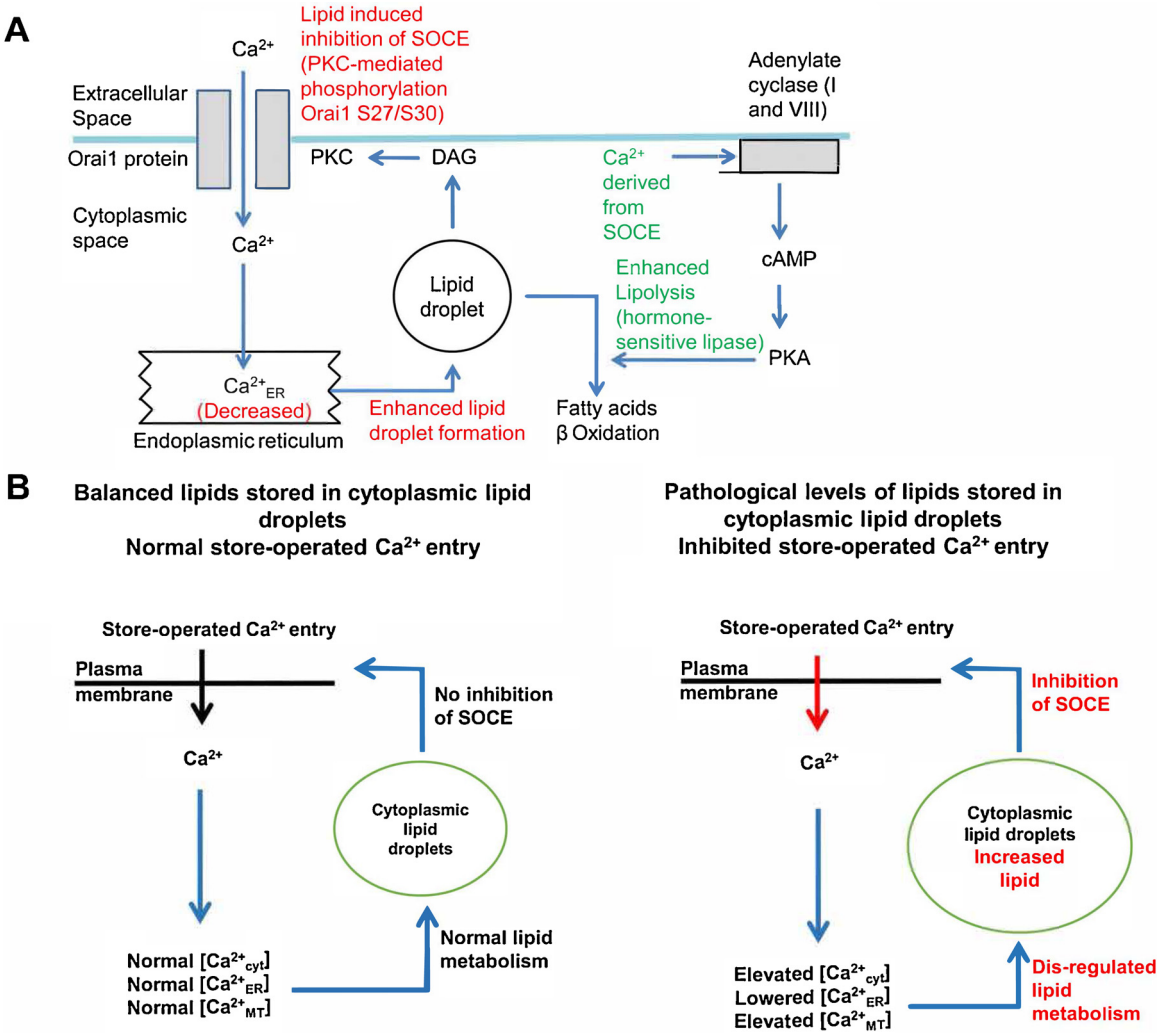

Fig. 6. Schematic representation of the role of store-operated Ca^{2+} entry (SOCE) in regulating the amount of lipid stored in cytoplasmic lipid droplets in normal and in steatotic hepatocytes. A. Schematic representation of the hypothesised roles of Ca^{2+}, diacylglycerol (DAG), PKC, and cyclic AMP, in regulating the amount of lipid which accumulates in cytoplasmic lipid droplets in steatotic hepatocytes. It is proposed that an increase in lipid in cytoplasmic lipid droplets results in: DAG-activation of protein kinase C (PKC), phosphorylation of the store-operated Ca^{2+} channel protein, Orai1, inhibition of store-operated Ca^{2+} entry (SOCE), and subsequent decreases in cyclic AMP and lipolysis, which then exacerbate the accumulation of lipid. B. Schematic representation of the proposed positive feedback cycle involving SOCE and lipid in cytoplasmic lipid droplets. Lipid inhibition of SOCE leads to altered intracellular Ca^{2+} homeostasis, increased lipid synthesis, and decreased lipid hydrolysis and autophagy, thus enhancing further lipid accumulation and further SOCE inhibition.

seem counterintuitive. However, it is possible that a chronic depletion of ER Ca^{2+} stores in lipid-loaded hepatocytes due to a reduced activity of SERCA2b leads to a chronic activation of low-level SOCE which contributes to the chronic elevation of $[Ca^{2+}]_{cyt}$.

It is worth noting that there are relatively few reported direct measurements of the basal values of $[Ca^{2+}]_{cyt}$ in steatotic compared to normal hepatocytes (Table 1). Comparison of basal $[Ca^{2+}]_{cyt}$ between control and lipid-loaded liver cells, measured using fluorescent Ca^{2+} reporters, such as Fura-2, and fluorescence imaging, requires stringent calibration of the fluorescence signals in order to derive the correct absolute value of $[Ca^{2+}]_{cyt}$ for each set of the cultured cells, or at least an accurate ratio for a comparison of the basal $[Ca^{2+}]_{cyt.}$. This is because the value for the dissociation constant for the binding of Ca^{2+} and the fluorescence efficiency of the reporter depend on the environment in which the reporter is located, and can also influence the capacity of the reporter to buffer Ca^{2+}. Relevant environmental factors include pH, ionic and protein composition, ionic strength, and the presence or absence of lipids [84–86]. These calibration issues are less important for measurements conducted on the same coverslip where the cells are subjected to a series of interventions, for example the experiment shown in Fig. 5A.

The results of each of the studies reported in Table 1 represent information obtained for one or more selected time points in the sequence of events from the onset of simple steatosis to the development of NASH, fibrosis, possibly cirrhosis, and ultimately HCC in the livers and/or liver cells in the selected model under investigation. During this continuum of pathological changes, lipid-induced changes in intracellular Ca^{2+} transporters and channels may change in nature and/or degree over time. Therefore, it seems important to interpret the observations summarised in Table 1 in this context. It would be valuable to have more information on how the parameters listed in Table 1 change during the progression of simple steatosis to the development of HCC. This might be particularly important for changes in $[Ca^{2+}]_{MT}$ which, as discussed above, so far seem only partially defined.

6. Lipid-induced inhibition of store-operated Ca^{2+} homeostasis and deranged intracellular Ca^{2+} homeostasis exacerbate lipid accumulation in hepatocytes

Several different experimental approaches have provided evidence

Table 2
Proposed mechanisms for the exacerbation of lipid accumulation in steatotic hepatocytes arising from altered hepatocyte Ca^{2+} homeostasis.

Alteration in Ca^{2+} homeostasis	Proposed mechanism	References
Decreased store-operated Ca^{2+} entry	Inhibition of lipolysis. Initiated by low SOCE leading to decreased formation cyclic AMP at the plasma membrane, decreased PKA, and decreased lipase activity	[12]
Increased $[Ca^{2+}]_{cyt}$	Inhibition of autophagy of cytoplasmic lipid droplets Initiated by elevated $[Ca^{2+}]_{cyt}$.	[122] [13]
Increased $[Ca^{2+}]_{MT}$	Inhibition of beta-oxidation pathway. Initiated by elevated $[Ca^{2+}]_{MT}$ leading to mitochondrial damage and dysfunction.	[51] [55]
Decreased $[Ca^{2+}]_{ER}$	Activation of lipid synthesis and inhibition of lipid secretion from hepatocytes. Initiated by low $[Ca^{2+}]_{ER}$ and mediated by ER stress and SREBP	[46] [9]

that altered intracellular Ca^{2+} homoeostasis causes and/or exacerbates lipid accumulation in hepatocytes, as well as in other cell types. The main initiating change in Ca^{2+} transporters, channels, and Ca^{2+} concentrations together with the proposed mechanisms are summarized in Table 2. These involve decreased lipolysis, inhibition of lipid autophagy, inhibition of beta-oxidation, increased lipogenesis and decreased secretion of lipids to the blood.

In experiments conducted with normal (non steatotic) hepatocytes incubated in the presence of exogenous fatty acids, pharmacological inhibition of SOCE, or knockdown of Orai1 and STIM1 using siRNA, were each found to cause a 2-fold enhancement of lipid accumulation [7]. These observations suggested that lipid-induced inhibition of SOCE exacerbates further lipid accumulation via a positive feed-back mechanism between SOCE and lipid accumulation, as shown schematically in Fig. 6B.

Based on an earlier study with *Drosophila* where it was shown that knockdown of STIM or other components of the SOCE pathway resulted in adipocity in the flies [87], and the results of many previous studies which have established that changes in intracellular Ca^{2+} regulate lipid metabolism, Maus and colleagues investigated the effects of inhibition of SOCE on lipid accumulation in various animal tissues and cells [88]. Employing a tamoxifen-inducible mouse model in which the STIM1 or STIM2 genes can be deleted to abolish SOCE, they showed that inhibition of SOCE leads to a large increase in lipid in skeletal and heart muscle and in liver. Moreover, they also found that cultured fibroblasts obtained from patients with loss-of-function mutations in Orai1 or STIM1, which abolished SOCE, exhibit an increase in lipid accumulation compared with controls. Further experiments provided evidence that in "normal" (non steatotic) fibroblasts and NIH3T3L-1 cells, SOCE is required to maintain mitochondrial fatty acid oxidation and lipolysis.

Results obtained from the measurement of cyclic AMP and lipolysis led to the proposal that SOCE provides a supply of intracellular Ca^{2+}, which activates adenylate cyclase VIII. This isoform of adenylate cyclase was previously shown to be located on the plasma membrane close to SOCE channels and to be activated specifically by Ca^{2+} derived from SOCE [89]. It was proposed that Ca^{2+} entry via the SOCE pathway leads to the generation of cyclic AMP, activation of PKA, activation of hormone sensitive lipase, and enhanced lipolysis [88] (shown schematically in Fig. 6A). Roles for Ca^{2+} and cyclic AMP in lipid metabolism are also supported by the findings that hepatic Ca^{2+}-activated adenylate cyclase III isoform plays a protective role in insulin resistance and obesity in mice on high fat diet [90], and that adenylate cyclase III haploinsufficiency contributes to obesity and insulin resistance in mice [91].

As discussed by the authors, the observations and deductions of Maus and colleagues that knock down of SOCE is associated with a decrease in $[Ca^{2+}]_{MT}$ and decreased mitochondrial beta oxidation of fatty acids, and that increased $[Ca^{2+}]_{cyt}$ is associated with increased lipid autophagy differ somewhat from changes observed by several other laboratories (Table 2). These include lipid-induced increases in $[Ca^{2+}]_{MT}$ and lipid-induced decreases in beta oxidation due to Ca^{2+}-induced mitochondrial damage, as well as decreased lipid autophagy. Reasons for differences between observations and conclusions in these studies may include whether or not the cells employed were initially steatotic or non steatotic, differences in cell type and treatment (eg knockdown of SOCE in non-lipid-loaded cells compared with effects of lipid on SOCE), and differences in time of exposure to lipid.

As described above, Ca^{2+} signaling pathways contribute to the regulation of lipid metabolism and storage in hepatocytes under normal physiological conditions (summarized schematically in Figs. 1 and 6B). The flow of fatty acids to hepatocytes and lipid storage in cytoplasmic lipid droplets are balanced by lipid metabolism needed for ATP synthesis and biosynthetic pathways. The concentrations of Ca^{2+} in the lumen of the ER and the cytoplasmic space and ER functions are all normal, and there is minimal formation of ROS. However, under conditions where the liver is the recipient of excess fatty acids, as in NAFLD, the normal "equilibrium" or "steady-state" of both Ca^{2+} homeostasis and lipid storage are substantially perturbed in favor of accumulation of high levels of lipid, ER stress, and greatly increased production of ROS. This "pushes" the normal balance towards the development of NASH and the promotion of HCC. In this sense, SOCE (and other Ca^{2+} channels and transporters) could be viewed as "sensors" of the amount of lipid accumulated in hepatocytes. Thus, as shown schematically in Fig. 6B, as lipid accumulates in hepatocytes, SOCE is inhibited leading to changes in $[Ca^{2+}]_{cyt}$, $[Ca^{2+}]_{ER}$ and $[Ca^{2+}]_{MT}$ which in turn lead to further lipid accumulation and further inhibition of SOCE.

7. Deranged intracellular Ca^{2+} homeostasis in steatotic hepatocytes creates an environment which could promote the development and progression of hepatocellular carcinoma

As discussed above, chronically elevated lipid in steatotic hepatocytes of NAFLD subjects is a major risk factor for the development of HCC [2,23]. Animal studies, principally with mice and rats, have shown that a high fat diet enhances the development of HCC, although the nature and extent of HCC depends on the agent employed to induce mutagenesis [92–96]. A recent study involving a mouse model of steatohepatitis has provided evidence that incorporation of cholesterol into a high fat diet enhances the induction of HCC by diethylnitrosamine (DEN) [16]. The results of numerous studies provide evidence which suggests that chronic steatosis and lipid-induced changes in intracellular Ca^{2+} concentrations in hepatocytes provide an environment in the liver which favors the generation of HCC mutations, promotes the rapid growth of cells expressing these mutations, and inhibits apoptosis [2,16,29,40,43]. The key components of this HCC promotional environment created by deranged intracellular Ca^{2+} homeostasis include exacerbated accumulation of lipids, lipotoxicity, ER stress, elevated ROS, and the activation of Ca^{2+}calmodulin dependent kinases and transcription factors which enhance mutagenesis, enhance cell growth, and lead to insulin resistance and type 2 diabetes (Fig. 1 and Table 3).

Two studies involving interventions in Ca^{2+} signaling pathways have provided evidence that elevated $[Ca^{2+}]_{cyt}$ in mouse livers can lead

Table 3
Proposed mechanisms through with altered hepatocyte Ca^{2+} homeostasis in non alcoholic fatty liver disease could create an environment for the development and promotion of hepatocellular carcinoma.

Parameter affected by lipid-induced alterations in hepatocyte Ca^{2+} homeostasis	Proposed mechanism leading to HCC development	References
Increased hepatocyte lipids (principally in cytoplasmic lipid droplets): both amount and nature of lipids	High concentration of toxic free fatty acids and other lipids ("lipotoxicity"). Lipids provide a substrate for generation of ROS	[44]
ER stress and the ER stress response	Inflammatory mediators and caspase-2	[123]
Increased ROS (oxidative stress)	Promotion of HCC development via activation of STAT-3	[15]
	Promotion of mutagenesis via chronic induction of transcription factor Nrf2 which promotes the progression of HCC	[99,101] [100] [102,98]
Increased $[Ca^{2+}]_{cyt}$	Promotion of insulin resistance and type 2 diabetes (risk factors for HCC) via activation of CaMKII and inhibition of Akt phosphorylation	[110] [124] [111]
	Promotion of growth of cancer cells via activation of CamKK2, CaMKIV and S6 kinase.	[14]
Decreased SERCA2b activity	Increased $[Ca^{2+}]_{cyt}$ Increased ROS	[9]
Decreased SOCE	Promotion of insulin resistance (risk factor for HCC) possibly via altered O-GycNAcylation of STIM1.	[11]

to the development of HCC [9,14]. In experiments which employied hepatic cancer cell lines and a mouse model of HCC Lin et al. showed that expression of CaMKK2 is increased in HCC. The degree of upregulation correlated inversely with HCC survival (Fig. 7) [14]. Moreover, knock down of CaMKK2 inhibited cancer growth. It was proposed that chronically elevated $[Ca^{2+}]_{cyt}$ activates CaMKK2 which in turn interacts with CaMKIV and the mTOR (target of rapamycin) complex, leading to activation of protein synthesis and the growth of

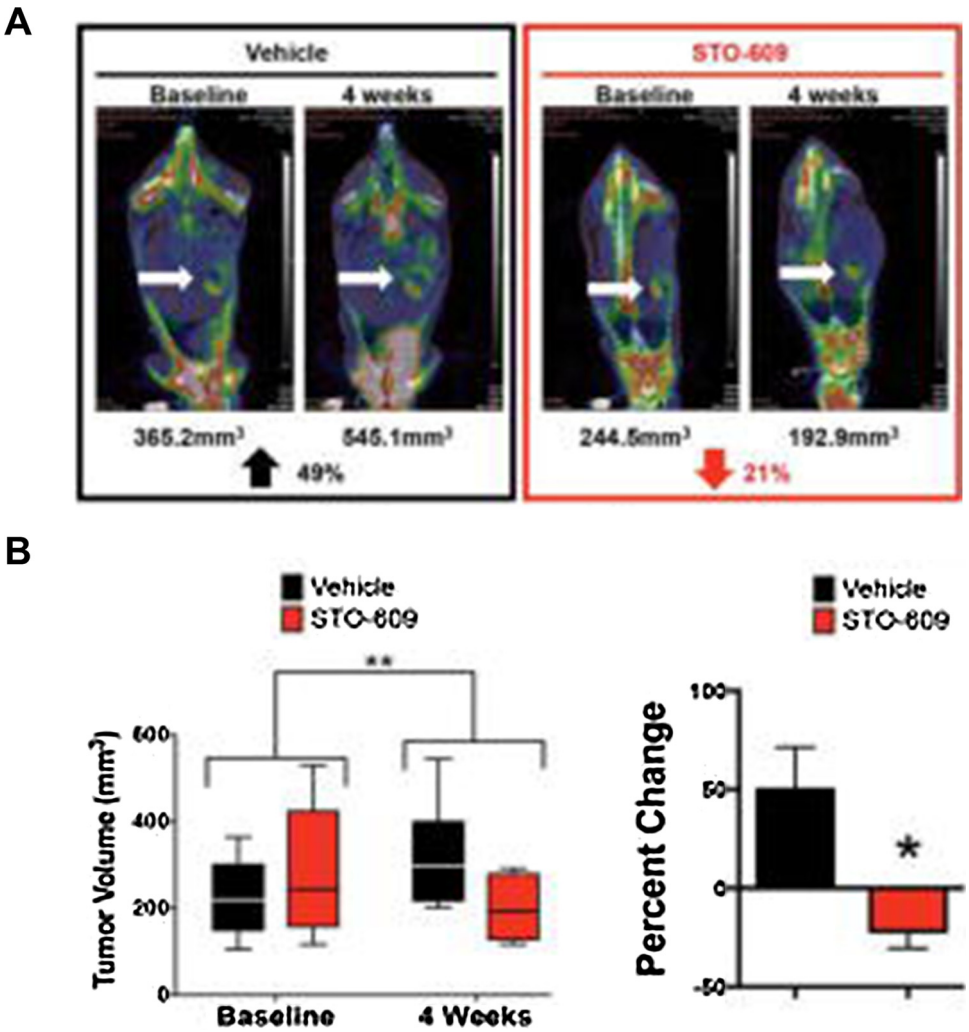

Fig. 7. Pharmacological inhibition of CaMKK2 using STO-609 reduces tumor growth in a mouse model of HCC in which mutations are induced by DEN. A. PET/CT images of liver tumors in mice pre-treated with DEN for 6 months to induce HCC tumors, then subjected to 4 weeks administration (by i.p. injection) of the CaMKK2 inhibitor STO-609, or vehicle. White arrow indicates the location of a DEN-induced liver tumor. B. Quantitation of tumor volume. (From Lin et al. 2015 [14]).

Table 4
Potential targets involving Ca^{2+} signaling pathways for therapeutic intervention directed to prevention of development of hepatocellular carcinoma in non alcoholic fatty liver patients.

Proposed Ca^{2+} transporter, channel, or Ca^{2+} binding protein	Proposed intervention strategy	References
SERCA2b	Activation using small molecule activator such as the allosteric activator CDN1163	[111]
	Activation by modification of ER membrane fluidity affected by altering thioesterase superfamily member 2/phosphatidyl transfer protein	[8]
	Increased expression induced by Maresin 1 leading to increased AMPK activity	[125]
	Activation by modulation of the SERCA2b regulator protein Cisd2	[9]
Ca^{2+} entry	Activation of SOCE by small molecule activator of Orai1 or STIM1.	[126]
	Inhibition of PKC leading to de-phosphorylation and activation of Orai1	[12]
	Inhibition of Ca^{2+} entry using Ca^{2+} channel blockers verapamil and nifedipine	[13,114]
Ryanodine receptors (RYR1 and RYR2)	Activation using small molecules such as caffeine and caffeine analogues	[16]
$InsP_3R$	Inhibition using small molecule inhibitors such as heparin and caffeine	[16,49]
CaMKII	Inhibition by natural product tetrandrine of phosphorylation of CaMKII	[124]
CaMKK2	Inhibition using small molecule inhibitor such as STO-609	[14]

mutated cells.

Evidence that inhibition of SERCA2b can lead to HCC has come from the studies of Cisd2 haploinsufficiency described above [9]. Using Cisd2 insufficiency as a strategy to induce inhibition of SERCA2b, it was shown that mice homozygous for CISD2 (in which liver SERCA2b is inhibited) exhibit a higher incidence of HCC induced by HBV or DEN [9]. The authors proposed a sequence of events in which inhibition of SERCA2b leads to increased $[Ca^{2+}]_{cyt}$, increased $[Ca^{2+}]_{MT}$, increased ROS, increased mitochondrial damage and ultimately development of HCC.

Probably the single most important factor which mediates the proposed link between lipid-induced alterations in hepatocyte Ca^{2+} homeostasis and the promotion of HCC is the generation of ROS (oxidative stress) together with associated chronic inflammation [15,44,97]. Elevated ROS is likely initiated by elevated $[Ca^{2+}]_{MT}$, decreased $[Ca^{2+}]_{ER}$ and ER stress. and these changes are sustained by exacerbated lipid accumulation [9,14,15,98–102]. Several studies have provided evidence that ROS and ER stress *per se* can lead to HCC in mouse and rat livers [103–107].

Recently, Grohman and colleagues have shown that one pathway initiated by oxidative stress is the activation of T-cell protein tyrosine phosphatase (TCPTP) which in turn activates STAT-3 phosphatase leading to the promotion of HCC [15]. Chronically elevated ROS in steatotic hepatocytes has been shown to activate the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) which is an important mediator of the development of HCC [99]. Under conditions of acute oxidative stress, such as in ischemia reperfusion of the liver associated with liver surgery, Nrf2 is activated and induces the synthesis of heme oxygenase-1 and other protective antioxidant enzymes [108]. Several laboratories have provided evidence that, under conditions of sustained oxidative stress, as occur in NAFLD and NASH, chronic elevation of Nrf2 inhibits ROS-induced cell death of hepatocytes which have already accumulated oncogenic mutations, thereby allowing these cells to accumulate further mutations [98–101]. It is interesting to note that knock out of Orai1 or STIM1, and hence SOCE, in mouse embryonic fibroblasts renders the cells more susceptible to damage by oxidative stress [109]. This susceptibility is associated with increased translocation of Nrf2 to the nucleus and activation of Nrf2. These observations suggest that one additional consequence of lipid-induced inhibition of SOCE is to contribute to mechanisms which cause chronic activation of Nrf2 and subsequently enhance the development of HCC.

Another avenue by which altered Ca^{2+} homeostasis could lead to promotion of HCC broadly involves the development of insulin resistance and type 2 diabetes, a risk factor for HCC [2,3]. Activation of CaMKII by chronically elevated $[Ca^{2+}]_{cyt}$ in steatotic hepatocytes has been shown to inhibit insulin signaling leading to insulin resistance [110]. On the basis of studies involving an obese mouse model and isolated mouse hepatocytes, it was proposed that activation of CaMKII by chronically elevated $[Ca^{2+}]_{cyt}$ in steatotic hepatocytes leads to ER stress via the downstream signaling proteins p38 and MK2. This in turn attenuates insulin action by inhibiting insulin-induced phosphorylation of Akt [110]. More recently, a second pathway by which chronically elevated $[Ca^{2+}]_{cyt}$ in steatotic hepatocytes is thought to inhibit insulin signaling has been identified [111]. This involves the inhibition by elevated $[Ca^{2+}]_{cyt}$ of the binding of Akt and other pleckstrin homology (PH) domain proteins to the cytoplasmic side of the plasma membrane, inhibition of Akt phosphorylation and impaired insulin signaling.

While two of the studies described above, knockout of CamKK2 and SERCA2b, provide evidence that changes in intracellular Ca^{2+} homeostasis in hepatocytes directly leads to, or can "cause" HCC [9,14], more research is needed to test the ideas described in Fig. 1 and the proposed mechanisms and links listed in Table 3. Thus, the idea that lipid-induced alterations in hepatocyte Ca^{2+} homeostasis creates an environment which promotes the development of HCC is presently based principally on hypothesized links between a number of separate observations. For example, the sequence of steps leading from altered intracellular Ca^{2+} homeostasis to the generation of ROS, enhancement of mutagenesis and/or increased cell proliferation and decreased apoptosis and HCC.

8. Hepatocyte Ca^{2+} channels and transporters as potential therapeutic targets in preventing the development of hepatocellular carcinoma in non alcoholic fatty liver disease

The information presented above indicates that altered hepatocyte intracellular Ca^{2+} homeostasis leads to intracellular and intra organ environments which could promote the development of HCC. This suggests that normalization of intracellular Ca^{2+} in the hepatocytes of NAFLD patients may reduce the likelihood of NAFLD proceeding to NASH and HCC. Therefore, pharmaceutical interventions which could normalize hepatocyte intracellular Ca^{2+} homeostasis in NAFLD are of potential value. Table 4 lists Ca^{2+} transporters, channels and Ca^{2+} binding proteins in hepatocytes which have been identified as, or implied to be, potential drug targets for pharmaceutical intervention directed towards reducing liver lipid and preventing HCC. Future clinical use of a given pharmacological intervention would depend on being able to identify those NAFLD patients who are likely to progress to HCC. While knowledge of prognostic markers is growing, further research is still needed [27].

Two examples of potential "proof of principle" for pharmacological intervention strategies involve the activation of SERCA2b. Administration by intraperitoneal (i.p.) injection of CDN1163, a SERCA2b allosteric activator, to genetically obese mice (ob/ob) has been shown to reduce liver steatosis [112]. In addition, CDN1163 reduced ER stress, improved control of blood glucose by insulin, and

decreased expression of genes encoding enzymes involved in lipid and glucose synthesis, induced apoptosis and induced biogenesis of mitochondria. From these results it might be predicted that small molecule activation of SERCA2b would also reduce development of HCC by decreasing liver lipid and preventing insulin resistance and type 2 diabetes. Evidence that activation of SERCA2b does reduce development of HCC has been provided by the studies of Cisd2 haploinsufficiency [9]. Overexpression of Cisd2 in mice with Cisd2 haploinsufficiency, which activated SERCA2b, suppressed the development of HCC induced by hepatitis B virus or DEN.

Interventions involving Ca^{2+} calmodulin activated kinases have also been shown to decrease the incidence of HCC. As part of their study, mentioned above, of the roles of CaMKK2 and CaMKIV in mediating the development of HCC initiated by elevated $[Ca^{2+}]_{cyt}$ in hepatocytes, Lin and colleagues found that decreased expression of the Ca^{2+} binding protein CaMKK2, or pharmacological inhibition of this kinase with STO-609, decreased HCC induced by DEN in a mouse model (Fig. 7) [14].

As listed above in Table 2, there is evidence that, in steatotic hepatocytes, chronic elevation of $[Ca^{2+}]_{cyt}$ inhibits lipid autophagy and thus contributes to steatosis. Park and colleagues have investigated the use of Ca^{2+} channel blockers with the aim of decreasing $[Ca^{2+}]_{cyt}$ and increasing lipid autophagy, and hence reducing lipid levels in steatotic hepatocytes [113]. Using HepG2 liver cells, they showed that incubation of the cells with saturated fatty acids increased $[Ca^{2+}]_{cyt}$ and decreased lipid autophagy. They also provided evidence that lipid-induced inhibition of SERCA2b *per se* (rather than ER stress, which is a consequence of SERCA2b inhibition) is responsible for lipid-induced inhibition of autophagy. The palmitate-induced increase in $[Ca^{2+}]_{cyt}$ in HepG2 cells was greatly reduced by co-incubation of the cells with the VOCC blockers verapamil (a phenylalkylamine) or nicardipine (a dihydropyridine). Further studies with mice fed a high fat diet showed that the administration of verapamil during the period of the high fat diet reduced liver lipid and insulin resistance, and decreased basal $[Ca^{2+}]_{cyt}$ measured in isolated hepatocytes. A subsequent study, which employed the dihydropyridine VOCC blocker nifedipine, presented as nifedipine-loaded nanoparticles, HepG2 cells, and a mouse model of NAFLD, demonstrated that the sustained release of nifedipine decreased lipid by enhancing lipid autophagy, and decreased insulin resistance [114].

While the results of these studies are potentially valuable in terms of developing strategies for the redection of liver lipid, the mechanisms by which VOCC blockers reduce liver lipid may need further investigation. Thus, as summarised in Section 5 above, evidence for the presence of functional VOCCs in normal adult rat and mouse hepatocytes is somewhat limited. HepG2 cells, in which expression of VOCC channel subunits was detected, are de-differentiated hepatocytes derived from human HCC and hence may not completely reflect the characteristics of normal human hepatocytes, and, at appropriate concentrations, VOCC blockers can inhibit SOCE as well as other Ca^{2+} channels. Moreover, VOCCs are expressed in a number of other cell types present in liver, including stellate, Kupffer and smooth muscle cells. It is therefore possible that the beneficial effects of verapamil, nicardipine and nifedipne in reducing liver lipid are due to the actions of these agents on cells other than hepatocytes, including those of the liver vasculature. Nevertheless, the potential to use VOCC blockers, which have been in clinical use for treatment of cardiovascular disorders for a long time, repurposed to reduce liver lipid in NAFLD and potentially also to reduce development of HCC, is valuable.

SOCE also offers a potential target for pharmacological intervention to decrease liver lipid and reduce development of HCC. On the basis of the results described above, it can be predicted that the activation of SOCE in steatotic hepatocytes would also lead to the activation of adenylate cyclase, PKA, cytoplasmic lipases and lipolysis, thus decreasing lipid. Some indication that activation of SOCE in steatotic livers could lead to a decrease in hepatocyte lipid comes from the observation that overexpression of STIM1 in the livers of obese mice led to an increase in hepatocyte SOCE and improved glucose tolerance [11]. The simultaneous pharmacological activation of both SOCE and SERCA2b would also be predicted to restore $[Ca^{2+}]_{ER}$ and intracellular Ca^{2+} homeostasis to near normal levels, and reduce ER stress and ROS. Activation of SOCE in steatotic hepatocytes might be achieved using small molecule activators of Orai1, or by inhibiting PKC, which as described above, is thought to phosphorylate and inhibits Orai1 (Fig. 6A).

PKC inhibitors may also offer other advantages. PKCalpha is overexpressed in HCC and, in part, may promote the generation of ROS [115–117]. Inhibition of PKCalpha in liver cell lines reduces cell proliferation, migration and invasion and induces apoptosis [116,118]. While isoforms of PKC are involved in numerous signaling pathways in hepatocytes, selective inhibition of one or more PKC isoforms may be beneficial in reducing liver lipid and HCC.

9. Conclusions

Lipid-induced alterations in intracellular Ca^{2+} homeostasis in steatotic hepatocytes play important roles in HCC pathology, including exacerbation of lipid accumulation, generation of ER stress and ROS, activation of Ca^{2+} calmodulin-sensitive kinases and activation of transcription factors. Temporal changes in Ca^{2+} transporters, channels and intracellular Ca^{2+} concentrations during the progression of simple steatosis to NASH and HCC, and the mechanisms involved, are yet to be fully understood. The results of future studies should add to our understanding of the steps involved in the development of HCC, and to the identification of pharmacological interventions which can lower liver lipid in NAFLD and prevent development of HCC.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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