


Activation of the mitogen-activated protein kinase ERK1/2 signaling pathway suppresses the expression of ChREBP α and β in HepG2 cells

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The carbohydrate response element-binding protein (ChREBP), a glucose-responsive transcription factor that plays a critical role in the glucose-mediated induction of genes involved in hepatic glycolysis and lipogenesis, exists as two isoforms: ChREBP α and ChREBP β . However, the mechanism responsible for regulating the expression of both ChREBP α and β , as well as the mechanism that determines which specific isoform is more responsive to different stimuli, remains unclear. To address this issue, we compared the effects of several stimuli, including oxidative stress, on the mRNA and protein expression levels of ChREBP α and β in the hepatocyte cell line, HepG2. We found that H₂O₂ stimulation suppressed the expression of both mRNA and protein in HepG2 cells, but the mRNA expression level of ChREBP β was < 1% of that for ChREBP α levels. In addition, the reduction in both ChREBP α and β mRNA levels was reversed by PD98059, a selective and cell permeable inhibitor of the MEK/ERK pathway. Additionally, the administration of 12-O-tetradecanoylphorbol 13-acetate (TPA) and staurosporine (STS), activators of extracellular-signal-regulated kinase (ERK) signaling, also resulted in a decrease in the levels of both ChREBP α and β mRNA in HepG2 cells through ERK signaling. These collective data suggest that oxidative stress, including STS treatment, suppresses the expression of ChREBP α and β via the activation of ERK signaling in HepG2 cells. Such a decrease in the levels of expression of ChREBP α and β could result in the suppression of hepatic glycolysis and lipogenesis, and this would be expected to prevent further oxidative stress.

Reactive species, which mainly include reactive oxygen species (ROS), are products generated in mitochondria and in glucose metabolism in eukaryotic cells [1]. Excessive ROS levels are thought to be the major cause of macromolecular damage and have been implicated in the development of numerous diseases. ROS

production is strongly dependent on the mitochondrial respiratory chain, a system that is greatly influenced by upstream glycolysis [2,3]. Glycolysis, in turn, is also known to be significantly involved in oxidative stress by virtue of its ability to change the ratio of NAD⁺ to NADH [4]. Glycolysis is largely regulated by three

Abbreviations

ACC, acetyl-CoA carboxylase; AGEs, advanced glycation end products; ChREBP, carbohydrate response element-binding protein; ERK, extracellular-signal-regulated kinase; FAS, fatty acid synthase; GK, glucokinase; L-PK, Liver-type pyruvate kinase; MAPK, mitogen-activated protein kinase; Mlx, max-like protein; NAC, N-acetyl-L-cysteine; O-GlcNAc, O-linked N-acetylglucosamine; PFK, phosphofructokinase; PP2A/PKA, protein phosphatase 2A/protein kinase A; ROS, reactive oxygen species; SREBP, sterol regulatory element-binding proteins; STS, staurosporine; TPA, 12-O-tetradecanoylphorbol 13-acetate; TXNIP, thioredoxin-interacting protein.

enzymes, glucokinase (GK), phosphofructokinase (PFK), and liver-type pyruvate kinase (L-PK), which function as rate-limiting enzymes [5]. The L-PK promoter is positively regulated by the carbohydrate response element-binding protein (ChREBP), a member of the basic helix-loop-helix family of transcription factors and transactivating glucose-responsive genes by binding to DNA as a heterodimer with the Max-like protein (Mlx) at a well-defined carbohydrate-responsive element. This transcription factor has recently been reported to be a key determinant in controlling hepatic glycolysis and de novo fatty acid synthesis in the liver under physiological conditions [6].

The activation of ChREBP through nuclear/cytosol shuttling in response to metabolites derived from glucose is a subject that is an object of considerable interest. Briefly, inactive ChREBP is dephosphorylated by Xu-5-P/PP2A and is then translocated into the nucleus through interaction with importin α , thus inducing the transcriptional expression of L-PK and lipogenic genes. In addition, starving signals such as the glucagon driven nuclear export signal are dependent on PKA phosphorylation and complex formation with 14-3-3 [7–9]. In addition, our group previously reported that O-linked N-acetylglucosamine (O-GlcNAc) modification also increased the glucose response of ChREBP [10]. In contrast, few studies regarding the transcriptional regulation of ChREBP have appeared. It was reported that the liver x receptor (LXR)/retinoid x receptor (RXR) complex and the thyroid hormone receptor (TR) binds to the promoter of ChREBP and induces its expression in the liver and white adipose tissue (WAT) [11,12]. Aside from its role in the regulation of metabolism, recent studies have also shed light on nonmetabolic factors that trigger the transcriptional regulation of ChREBP. For example, ChREBP has been shown to mediate the glucose induced expression of the thioredoxin-interacting protein (TXNIP), which induces oxidative stress in pancreatic β cells [13]; and that the mTOR-regulated ChREBP/TXNIP pathway plays an important role in inhibiting diabetes stress in β cells for cell survival [14].

More recently, reports have emerged showing that a distinct cellular response to nutrients exists between ChREBP α and β , two isoforms of ChREBP that are transcribed from different promoters. In mouse adipose tissue, the glucose-mediated activation of ChREBP α induces the expression of ChREBP β isoform, which is located exclusively in the nucleus. The transcriptional activity of ChREBP β is 20-fold greater than that of ChREBP α at high glucose concentrations, indicating a feed-forward mechanism and that ChREBP β may be a more potent activator of ChREBP transcriptional target genes [15]. When mice were subjected to the fasting–

refeeding of high carbohydrate diets, ChREBP α was not induced, even though ChREBP β levels were increased by 10- to 20-fold [16]. In addition, Meng *et al.* [17] reported that HNF-4 α promoted the transcription of ChREBP in response to glucose and HNF-4 α and ChREBP α coordinately increased the transcription of ChREBP β . Whereas in diabetic mice, islet ChREBP β was increased, which downregulates ChREBP α via a negative feedback loop to ultimately limit the excessive glucose induced by the ChREBP α -mediated gene expression [18]. In another study, Chen *et al.* [19] reported that advanced glycation end products (AGEs) promoted the expression of both ChREBP α and β through increasing ROS levels in HepG2 cells, suggesting that oxidative stress may regulate the expression of ChREBP, but the mechanism responsible for this remains unknown. From the above, it would appear that the responsiveness of ChREBP varies depending on the tissue and the type of stimulus. Furthermore, in terms of their contributions for ChREBP-attributed metabolic functions, it will be crucial to define specific regulators of ChREBP α and β .

Studies have shown that oxidative stress can cause organ-specific and path-specific toxicity, and plays a key role in cell proliferation, tumor development, and glucose/lipid metabolism [20–23]. Furthermore, the exposure of cells to exogenous H₂O₂ mimics oxidative stress and leads to the activation of the mitogen-activated protein kinase (MAPK) [24]. The MAPK pathways consist of a family of protein kinases including extracellular-signal-regulated kinase (ERKs), c-jun N-terminal kinases (JNKs), and p38, which play a crucial role in signaling transduction from the cell surface to the nucleus. The ERK cascade is induced by a variety of extracellular agents including growth factors, and hormones as well as oxidative stress. ERK activation following H₂O₂ stimulation has been reported to be involved in cell survival following oxidative injury in several cell lines, and these effects can be abolished by ERK inhibitors and antioxidants N-acetyl-L-cysteine (NAC) [24–26].

This study focused primarily on HepG2 cells and investigated the effects of noncarbohydrate stimulation, especially oxidative stress, on the expression levels of ChREBP α and β . Based on our findings, we report on a novel mechanism by which the activation of the ERK signaling cascade suppresses the transcriptional expression of both ChREBP isoforms in hepatocytes.

Materials and methods

Chemicals

All chemicals used in this study were purchased from either Wako Pure Chemical Industries Ltd. (Osaka, Japan),

Nacalai Tesque, Inc. (Kyoto, Japan), Abcam Ltd. (Cambridge, UK), or Sigma-Aldrich, unless specified otherwise, and were of the highest grade available. Go6983 (PKC inhibitor) was purchased from Abcam (Cambridge, UK). 12-O-tetradecanoylphorbol 13-acetate (TPA) (PKC activator) and PD98059 (MEK inhibitor) were obtained from Cell Signaling Technology (Beverly, MA, USA). Staurosporine (STS) (a non-specific protein kinase inhibitor) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Thirty % H₂O₂ was purchased from Nacalai Tesque (Kyoto, Japan). NAC was obtained from WAKO (Osaka, Japan). STS was dissolved in ethyl acetate (WAKO, Osaka, Japan), and other compounds, unless otherwise specified, were dissolved in DMSO (WAKO, Osaka, Japan) and added directly to the culture medium.

Cell culture and treatment

Human hepatocyte carcinoma cell line HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum, 100 U·mL⁻¹ penicillin, and 100mg/ml streptomycin at 37 °C under an atmosphere of 95% air and 5% CO₂. The cells were plated in 6-well plates at a density of 2 × 10⁵·mL⁻¹ the day before treatment. Unless otherwise specified, the cells were pretreated with the indicated concentrations of Go6983 or PD98059 or NAC for 1 h, and then stimulated by H₂O₂/STS/TPA for an additional 24 h.

Western blotting analysis

HepG2 cells were washed with phosphate-buffered saline (PBS) twice and lysed by treatment with RIPA buffer (Fujifilm Pure Chemical Industries Ltd., Osaka, Japan). Cell lysates were centrifuged at 4 °C and 20 000 × *g* for 20 min. Supernatants were collected and quantified by means of a PierceTM BCA Protein Assay Kit (Thermo scientific USA Ltd., Rockford, IL, USA). Protein extracts were size-fractionated on a 5%–20% SDS/PAGE gel and transferred to a polyvinylidene fluoride difluoride (PVDF) membrane (GE Healthcare UK Ltd., Buckinghamshire, UK) in a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad USA Ltd., Redmond, WA, USA). The membrane was then incubated with the primary antibody (phospho-p44/42 MAPK (ERK1/2), anti-p44/42 MAPK (ERK1/2), anti- β -tubulin, and anti- β -actin (Cell Signaling Technology, Danvers, USA), 1 : 500; anti-PK (Gene Tex, Irvine, CA, USA), 1:500; anti-ChREBP (Novus Biologicals, NB400-135, USA), 1 : 500) and a secondary antibody Horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology, USA 1 : 500) overnight in an iBind Western System (Thermo scientific, Rockford, IL, USA), and proteins were visualized using an ECL Western Blotting Detection System (GE

Healthcare, Buckinghamshire, UK). Coomassie Brilliant Blue (quick CBB-plus, Wako, Osaka, Japan) staining was used as a loading control according to the manufacturer's instructions.

RNA extraction and reverse transcription

Total RNA was isolated from HepG2 cells using Sepazol RNA-I super G reagent (Nacalai Tesque, Kyoto, Japan). Total RNA (1 μ g) was reverse transcribed using a high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Real-time PCR

Real-time PCR was performed with the PowerTrackTM SYBR GreenMaster Mix (Thermo Fisher Scientific) and a Quant Studio 12K Flex Real Time PCR System (Applied Biosystems) with gene-specific primers or subjected to 2% agarose gel and then stained with ethidium bromide to detect the levels of mRNAs. The sequences of q-PCR primers (for human sapiens) are listed below.

ChREBP α	Forward (5'-ACTCGGACT CGGACACAG AC-3')	Reverse (5'-AGGCT CAAGCACTC GAAGAG-3')
ChREBP β	Forward (5'-AGCGGATTC CAGGTGAGG-3')	Reverse (5'-TTGTTC AGCGCGATC TTGTC-3')
β -actin	Forward (5'-GTGGCATCC ACGAACTAC CTT -3')	Reverse (5'-GGACTC GTCATACTCC TGCTTG -3')
FAS	Forward (5'-AACCGGCTC TCCTTCTTCTC GACTT-3')	Reverse (5'-TCCGA GCGGCAGTAC CCATT-3')
ACC	Forward (5'-GCAGAATTTGT TACTCGCTTTGG-3')	Reverse (5'-ATTTTCATA AGACCACCTAC GGATAGAC-3')
L-PK	Forward (5'-AATCCAGGTTT GGCTGGTT-3')	Reverse (5'-AGCACGA GTTGTGAAGA CAGT-3')
PFK	Forward (5'-CCACCGTGGAC TGTGTCTGTT-3')	Reverse (5'-GGGCACT CGCCGATTA GGTAT-3')
GK	Forward (5'-CAACTGGACCA AGGGCTTCAA-3')	Reverse (5'-CGTGGCCA CCGTGTCA TTC-3')

Data presentation and statistical methods

Data are expressed as the mean \pm SE from a single experiment representative of more than three independent experiments. Statistical analyses were performed with GraphPad Prism 6 using a Student's *t* test or one-way ANOVA. In all analyses, a *P* value of *P* < 0.05 was considered significant.

Results and Discussion

H₂O₂ treatment downregulates the gene expression of ChREBP α and β in HepG2 cells

To determine the effect of oxidative stress on the gene expression of ChREBP, HepG2 cells were challenged with 1 mM of H₂O₂ for 24 h and the mRNA levels of ChREBP α and β were analyzed by comparative analysis of agarose gel electrophoresis. As shown in Fig. S1, we observed that the mRNA levels of ChREBP α were much higher than those of ChREBP β and that the stimulation with 1 mM H₂O₂ reduced both the ChREBP α and β mRNA expressions. The real-time PCR analysis clearly showed that the addition of H₂O₂ suppressed the mRNA levels of both ChREBP α (Fig. 1A, left) and β (Fig. 1A, right) in a dose-

dependent manner and that the mRNA levels of ChREBP β were < 1% of the former in HepG2 cells (Fig. 1A, right). We therefore conclude that ChREBP α is the major isoform in HepG2 cells. In addition, stimulation with 1 mM H₂O₂ reduced the ChREBP α and β mRNA expression in a time-dependent manner (Fig. 1B). Further analysis by western blotting also showed that the protein expression level of ChREBP was decreased as the result of the H₂O₂ treatment (Fig. 1C). These results demonstrate that oxidative stress efficiently reduces the expression of both ChREBP α and β . Since the expression level of ChREBP β was quite low in the liver, the effect was expected to be extremely small compared to that of ChREBP α . Thus, ChREBP α appears to be a more potent mediator of hepatic ChREBP functions.

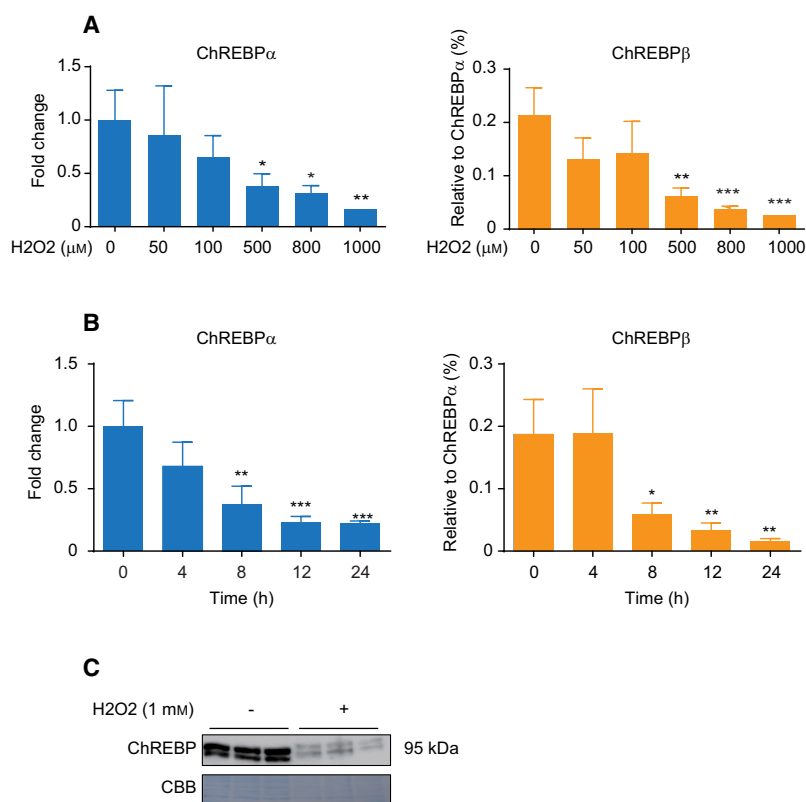


Fig. 1. Effect of ROS stress on ChREBP α and β expression. (A) HepG2 cells were exposed to indicated concentrations of H₂O₂ for 24 h. The mRNA levels of ChREBP α and β were measured by quantitative RT-PCR, respectively. Left panel shows the relative expression level of ChREBP α after normalization to β -actin. The right panel shows the relative expression level of ChREBP β normalized by ChREBP α . (B) HepG2 cells were incubated with 1 mM H₂O₂ for indicated hours. The mRNA levels of ChREBP α and β were measured by quantitative RT-PCR. Left panel shows the relative expression level of ChREBP α after normalization to β -actin. The right panel shows the relative expression level of ChREBP β normalized by ChREBP α . Data from one representative experiment of three independent experiments are presented as the mean \pm SE of three technical replicates. (A–B) Student *t* test: **P* < 0.05; ***P* < 0.01; ****P* < 0.001 versus normal group. (C) HepG2 cells were treated with or without 1 mM H₂O₂ for 24 h. The expression levels of ChREBP were detected by western blotting. A representative Coomassie Blue-stained blot is shown as a loading control.

ERK activation is required in H₂O₂ suppressed expression of ChREBP in HepG2 cells

To gain a deeper insight into the effects of the H₂O₂ treatment on the regulation of ChREBP, the involvement of ERK signaling was evaluated. When HepG2 cells were exposed to 1 mM H₂O₂ for the indicated number of minutes, a dramatic activation of ERK was observed after the cells had been exposed to H₂O₂ for 15 min and this activation increased with time as detected by western blotting (Fig. 2A). Pretreatment with PD98059, but not Go6983, specific inhibitors of the MAPK (MEK) and PKC, respectively, abolished the effect of H₂O₂ stimulation on the expression of both ChREBP α and β , suggesting that PKC-independent ERK activation was responsible for the reduced expression of ChREBP α and β in response to exposure to H₂O₂ (Fig. 2B). On the other hand, the addition of the antioxidant NAC restored the expression of ChREBP α but not that of β caused by the H₂O₂ treatment (Fig. 2B). Therefore, these results indicate that ROS including H₂O₂ negatively regulates the expression of both ChREBP α and β . On the contrary, Chen *et al.* [19] recently proposed an up-regulation of ChREBP α and β promoted by AGEs as the result of increasing ROS levels in HepG2 cells. However, it is unclear whether ROS resulting from AGEs activates

the expression of ChREBP α and β because glucose increases the expressions of ChREBP α and β , and AGEs contain glucose-like moieties. In light of these data, ChREBP is at least a potential candidate gene that is affected in response to oxidative stress, and our results provide direct evidence to show that ChREBP α and β is suppressed by oxidative stress in liver cells. Some studies have reported that the activation of ERK by H₂O₂ plays a critical role in cell survival following oxidative damage [24,26] and other studies have reported that ERK activation mediated by ROS induces apoptosis in a variety of cells [27,28]. Therefore, further study will be required to analyze the markers that are involved in cell survival or cell death regarding ChREBP following H₂O₂ exposure.

TPA treatment downregulates mRNA level of ChREBP through ERK activation in HepG2 cells

To ascertain the downregulation of ChREBP by ERK activation, we used TPA, which induces the PKC-dependent activation of ERK in HepG2 cells [29]. Similar to the effect of H₂O₂ exposure, the TPA treatment also reduced the mRNA levels of ChREBP α and β in a dose-dependent manner in HepG2 cells (Fig. 3A). In

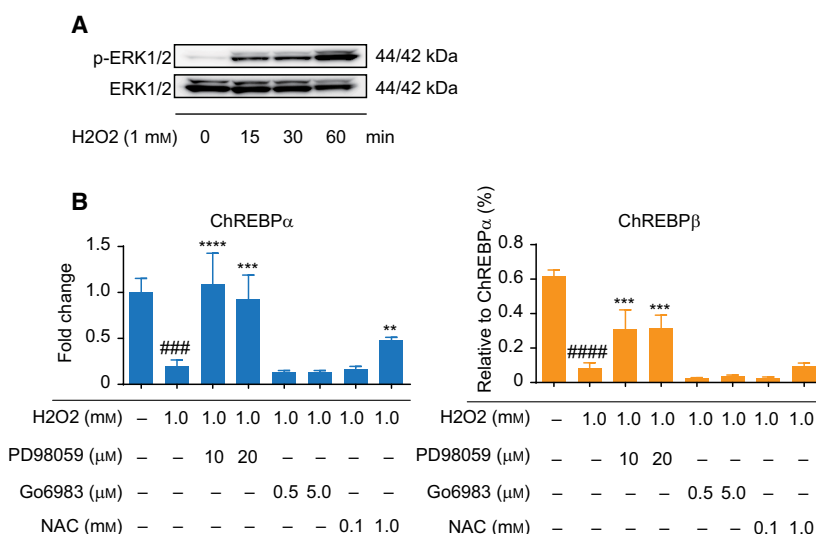


Fig. 2. Activation of p-ERK induced by H₂O₂ and the recovery of ChREBP α and β expression by an inhibitor for ERK and NAC, an antioxidant. (A) HepG2 cells were exposed to 1 mM H₂O₂ for indicated minutes. The expression levels of ERK and p-ERK were detected by western blotting. (B) HepG2 cells were pre-incubated in the presence or absence of PD98059, Go6983 or NAC. After 1 h, cells were treated with 1 mM H₂O₂ and incubated for additional 24 h. The mRNA levels of ChREBP α and β were measured by quantitative RT-PCR, respectively. Left panel shows the relative expression level of ChREBP α after normalization to β -actin. The right panel shows the relative expression level of ChREBP β normalized by ChREBP α . Data from one representative experiment of three independent experiments are presented as the mean \pm SE of three technical replicates. One-way ANOVA test: ### P < 0.001; ##### P < 0.0001 versus normal group. ** P < 0.01; *** P < 0.001; **** P < 0.0001 versus the H₂O₂ group.

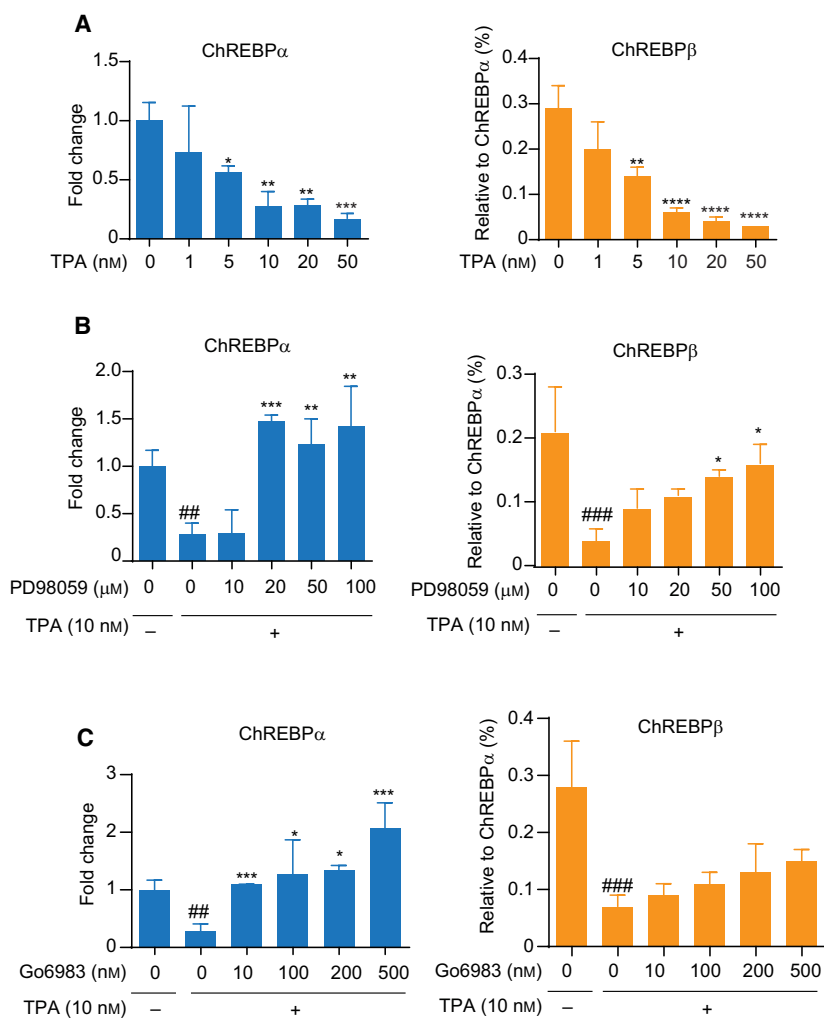


Fig. 3. Suppression of mRNA expression of ChREBP α and β by treatment with TPA in HepG2 cells. (A) HepG2 cells were treated with indicated concentrations of TPA for 24 h. The mRNA levels of ChREBP α and β were measured by quantitative real-time PCR, respectively. Left panel shows the relative expression level of ChREBP α after normalization to β -actin. The right panel shows the relative expression level of ChREBP β normalized by ChREBP α . Data from one representative experiment of three independent experiments are presented as the mean \pm SE of three technical replicates. Student *t* test: **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001 versus normal group. (B and C) HepG2 cells were pretreated with PD98059 and Go6983 at the indicated concentrations followed by treatment of 10 nM TPA for 24 h. The mRNA levels of ChREBP were measured by quantitative real-time PCR. Left panel shows the relative expression level of ChREBP α after normalization to β -actin. The right panel shows the relative expression level of ChREBP β normalized by ChREBP α . Data from one representative experiment of three independent experiments are presented as the mean \pm SE of three technical replicates. One-way ANOVA test: ###*P* < 0.01; ####*P* < 0.001 versus normal group. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 versus TPA group.

addition, both a MEK inhibitor (PD98059) and a PKC inhibitor (Go6983) completely restored ChREBP α expression levels and, at high concentrations, increased their expression somewhat (Fig. 3B left and C left). In contrast, ChREBP β was only partially restored by the ERK inhibitors (Fig. 3B right and C right) although the expression levels were measurably less than ChREBP α . These results demonstrate that PKC-dependent ERK activation suppresses the expression of ChREBP α .

STS suppresses expression of ChREBP through ERK activation in HepG2 cells

STS at lower concentrations is also an ERK activator [30,31] although, at high concentrations, it is also a well-known PKC inhibitor and apoptosis inducer. Thus, we next evaluated the effect of an STS treatment on ChREBP expression in a liver cell line. An initial

dose response of STS was tested in HepG2 cells. Cultured cells were exposed to from 0 to 10 nM STS since no obvious effects on cell proliferation or survival were observed at least for 48 h (data not shown). The mRNA expression levels of ChREBP α and β at 24 h after the STS treatment were decreased in a dose-dependent manner (Fig. 4A). When examined at a concentration of 10 nM, the expression levels of ChREBP α and β were decreased by the STS treatment in a time-dependent manner (Fig. 4B). To explore the mechanism responsible for the inhibition of STS on ChREBP expression, the role of ERK signaling was investigated. ERK was phosphorylated within 15 min by a 10-nM STS treatment (Fig. 5A) and the effect remained after a 24-h incubation period (Fig. 5B). Furthermore, pretreatment with PD98059 abolished the effect of STS on the gene expression of ChREBP α and β (Fig. 5C). The efficacy of PD98059 was further confirmed by western blotting. As shown in Fig. 5D,

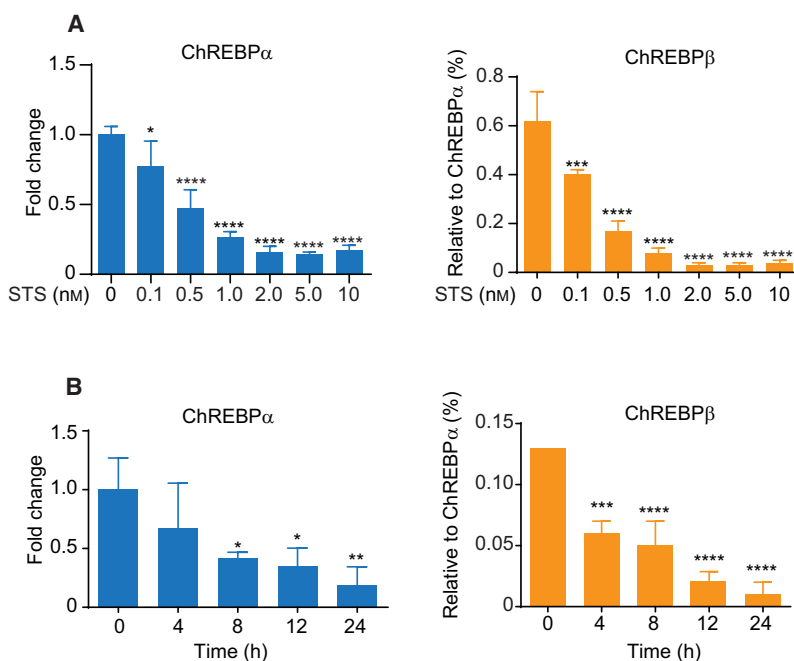


Fig. 4. Suppression of mRNA of ChREBP α and β by treatment with STS in HepG2 cells. (A) HepG2 cells were treated with indicated concentrations of STS for 24 h. The mRNA level of ChREBP α and β were measured by quantitative RT-PCR. Left panel shows the relative expression level of ChREBP α after normalization to β -actin. The right panel shows the relative expression level of ChREBP β normalized by ChREBP α . (B) HepG2 cells were challenged with 10 nM STS for indicated hours. The mRNA levels of ChREBP α and β were measured by quantitative RT-PCR. Left panel shows the relative expression level of ChREBP α after normalization to β -actin. The right panel shows the relative expression level of ChREBP β normalized by ChREBP α . Data from one representative experiment of three independent experiments are presented as the mean \pm SE of three technical replicates. (A–B) Student *t* test: **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001 versus normal group.

the administration of STS resulted in the phosphorylation of ERK and the level of phosphorylated ERK was significantly reduced by a PD98059 pretreatment. This result indicates that PD98059 substantially suppressed the activation of the MEK-ERK pathway caused by STS. However, pretreatments with Go6983 or NAC showed no effects on the STS elicited suppression of ChREBP α and β expression (Fig. 5E). Similar but different from the effect of H₂O₂, this implies that the STS triggered suppression of ChREBP expression also requires ERK activation, but that oxidative stress was not involved. A possible explanation for this is that a low concentration of STS is ineffective in generating oxidative stress in HepG2 cells. Furthermore, western blotting revealed that the protein expression level of L-PK, the downstream target gene of ChREBP, was reduced by the STS treatment (Fig. S2A). Quantitative analysis of the mRNA expression levels of the enzymes also showed significantly lower expressions of L-PK, fatty acid synthase (FAS), and acetyl-CoA carboxylase (ACC; Fig. S2B), which are consistent with the data observed in ChREBP-deficient mice [32]. These results suggest that glycolysis

and lipogenesis are also suppressed following ERK activation.

Taken together, we conclude that as long as ERK is activated by ROS, TPA, STS, etc., the expression of both ChREBP α and β is reduced, which indicates that the ERK pathway plays a prominent role in the transcriptional regulation of both isoforms, although the mRNA levels of ChREBP β are < 1% that of ChREBP α in HepG2 cells. Namely, the gene expression of ChREBP α is very sensitive to ERK signaling in liver cells. This investigation expands our current understanding of the response of ChREBP α and β to effectors other than carbohydrates and highlights a more potent role of hepatic ChREBP α rather than β in response to oxidative stress. As a result, the decrease in ChREBP α indicates that a reduced level of hepatic fatty acid synthesis and glycolysis may play a protective role in response to oxidative stress. Paradoxically, Sirek *et al.* reported that an insulin treatment increased the expression of ChREBP α through the Pak1/MEK/ERK/Oct-1 signaling cascade in mouse hepatocytes [33,34]. The insulin treatment promoted the expression of ChREBP α through ERK activation,

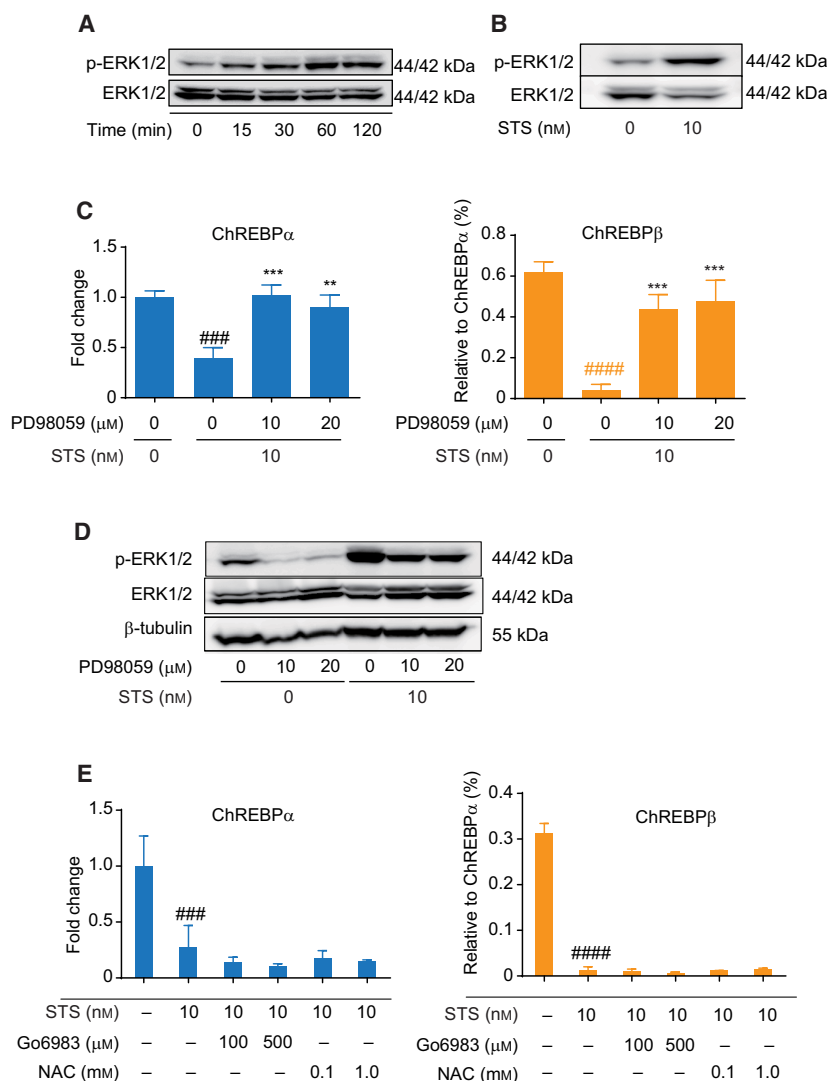


Fig. 5. ERK activation is required for the suppression of mRNA of ChREBP α and β by treatment with STS. (A and B) HepG2 cells were treated with 10 nM STS for 0–2 or 24 h. The expression levels of ERK and p-ERK were detected by western blotting. (C) HepG2 cells were pretreated with indicated concentrations of PD98059 and incubated additional 24 h with or without 10 nM STS. The mRNA levels of ChREBP α and β were measured by quantitative RT-PCR. Left panel shows the relative expression level of ChREBP α after normalization to β -actin. The right panel shows the relative expression level of ChREBP β normalized by ChREBP α . Data from one representative experiment of three independent experiments are presented as the mean \pm SE of three technical replicates. One-way ANOVA test: ### P < 0.001; #### P < 0.0001 versus normal group. ** P < 0.01; *** P < 0.001 versus STS group. (D) HepG2 cells were pretreated with indicated concentrations of PD98059 and challenged with or without 10 nM STS for additional 24 h. The expression levels of ERK and p-ERK were detected by western blotting. β -tubulin was used as an internal control. (E) HepG2 cells were pretreated with indicated concentrations of Go6983 or NAC and incubated additional 24 h with or without 10 nM STS. The mRNA levels of ChREBP α and β were measured by quantitative RT-PCR. Left panel shows the relative expression level of ChREBP α after normalization to β -actin. The right panel shows the relative expression level of ChREBP β normalized by ChREBP α . Data from one representative experiment of three independent experiments are presented as the mean \pm SE of three technical replicates. One-way ANOVA test: ### P < 0.001; #### P < 0.0001 versus normal group.

a process that involved the inactivation of oct-1, which, contrary to our findings, indicates that the activation of ERK suppresses ChREBP α and β expression. However, it is well known that ChREBP is primarily controlled via its cytosol-nuclei shuttle by

elevated glucose levels, while insulin was a potent inducer of sterol regulatory element-binding proteins (SREBP)-1c [35]. The issue of whether insulin can activate the expression of ChREBP independent of glucose stimuli remains controversial. We also attempted to

stimulate HepG2 cells with insulin, but no effect on the expression of ChREBP was detected (data not shown). Moreover, glucose activates the hepatic gene expression of ChREBP independent of insulin signaling although it can be activated by additional carbohydrates [36,37]. On the other hand, fructose administered by gavage robustly increased ChREBP β expression but not ChREBP α in the intestines of mice [38]. For the above reasons, specific isoforms of ChREBP should be studied separately when referring to any exogenous stimulus in different tissues. Further study will also be required to elucidate the mechanism of ChREBP expression at the transcriptional level including the involvement responsible for representative signaling pathways such as JNK and p38 induced by oxidative stress as well as ERK signaling.

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Conflict of interest

The authors declare no conflict of interest.

Data accessibility

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Author contributions

LL conceived and wrote the manuscript. LL, HS, and HE designed and carried out the experiments. NF and DY performed the statistical analyses. HS and KS analyzed the data. NF and HS assisted in writing the manuscript. All of the authors have read and approved the final version of the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Analysis of agarose gel electrophoresis of the ChREBP α and β mRNA expression by H₂O₂ treatment in HepG2 cells.

Fig. S2. Proteins and genes involved in glycolysis and lipogenesis by STS treatment in HepG2 cells.